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STUDIES ON THE CORRELATION BETWEEN METABOLIC GRADIENTS, ELECTRICAL GRADIENTS, AND GALVANOTAXIS. II. GALVANOTAXIS OF THE BROWN HYDRA AND SOME NON-FISSIONING PLANARIANS

LIBBIE H. HYMAN
Hull Zoölogical Laboratory, University of Chicago

SOME time ago I tentatively suggested an explanation of galvanotaxis (Hyman, 1918; Hyman and Bellamy, 1922) to the effect that orientation in an electric current was related to the direction of the bioelectric currents present within the animal. In most of the lower organisms the anterior end is positively charged (negatively with reference to the galvanometer) and consequently, I proposed, this end turns toward the cathode when the current is passed. A similar idea was not wanting in the literature; e.g., Cohn and Barratt (1905). Later and independently, Robertson (1927) developed the same idea with reference to the galvanotropic reaction of the triclad *Polycelis nigra*.

My theory involved no assumption as to the mode by which the orientation occurred. Consequently, Moore's (1923) inference that cataphoresis constituted the mechanism of orientation has no basis in anything stated in my papers. It is obviously impossible for an animal the size of the earthworm to be oriented by cataphoresis. My general viewpoint upon this matter would be that the orientation in an electric current takes place by means of the regular neuromotor or ciliary mechanism of the animal, but that the posture assumed is controlled by the internal electric currents in the animal. I am not, however, interested in the mechanism of orientation; my

point was that there is a relation between these inherent currents and the galvanotactic attitude and orientation. I am unable to see that Moore's theory or similar theories derived from Loeb's work explain the U-shaped orientation characteristic of annelids.

I shall not here undertake to review the literature which has accumulated since my last communication, but merely to put on record certain additional observations. These were made with the assistance of Mr. Gerald N. Bench, who became interested in the question of galvanotaxis in a course in animal behavior which he took with Professor W. C. Allee. Mr. Bench conferred with me about his work; and since he had the apparatus in readiness, I suggested that we try out together certain forms whose behavior seemed to me interesting from the point of view of my theory. These forms were *Hydra* and two non-fissioning planarians. The apparatus employed was that devised by R. S. Lillie and W. Cattell (1925) in which the current is led first through zinc electrodes into vessels containing a saturated solution of zinc sulphate and then through agar blocks into the dish where the animals are to be tested. This device seems to me the best yet suggested for testing galvanotactic response, since the products of electric dissociation cannot reach the experimental dish. In our experiments the experimental dish was an oblong composed of paraffin. This dish was 9 centimeters long and 4.6 centimeters wide. The depth of water during the tests was 1 centimeter. The amperage used was approximately 1 milliampere. The voltage was varied by means of a resistance coil; 5–20 volts are required to obtain a definite orientation.

I. HYDRA

The only recorded data on the galvanotaxis of *Hydra* are those of Pearl (1901), who states that the animal is anodic. When the current is passed, there is a contraction on the anodic side which causes a bending toward the anode and finally an orientation parallel to the current with the anterior end toward the anode. However, in Pearl's account there is recorded a certain amount of cathodic response. One is, in fact, left in some doubt as to the typical orientation. Now, according to my theory, *Hydra* should be cathodic; and consequently some of Pearl's observations constitute evidence against the correct-

ness of my idea. One notes, however, that Pearl worked with the green *Hydra*, and from the literature on galvanotaxis one gains the impression that green animals in general behave atypically. Thus green Protozoa tend to be anodic, while colorless ones are usually cathodic. Consequently it seemed desirable to try out some other species of *Hydra*. Large healthy specimens of the brown *Hydra* (*Pelmatohydra oligactis*) were at hand. These were put in water in the paraffin dish and left overnight to become attached. The following day the tests were made. Two different lots of animals were tried; the following is an abstract from the notebook.

In the first lot there were six animals. At 5 volts there was no definite response. At 8 volts three specimens showed some cathodic orientation. At 15 volts, three which were hanging to the surface film bent their anterior ends to the cathode. When the current was reversed, they also reversed. At 30 volts, three specimens which had become loose and were lying on the bottom, bent into a U-shape with anterior and posterior ends directed to the cathode. A fourth specimen fastened to the bottom turned the anterior end to the cathode, as did also one fastened to the surface film. The sixth animal gave no definite response. When the current was reversed, all five also reversed, bending in a U-shaped attitude toward the cathode.

In the second trial, eleven animals were present, eight fastened and three loose. At 10 volts, nine were cathodic, one indifferent, and one anodic. Upon reversals, a varying number, from five to eight, also reversed, while the others remained indifferent. At 20 volts, nine gave a very definite turning toward the cathode.

It is concluded that the brown *Hydra* (*Pelmatohydra oligactis*), when placed in a constant electric current of 8–20 volts, bends toward the cathode. Those lying loose assume a U-shape, with anterior and posterior ends pointing to the cathode; those that are attached bend, turning the whole body or the anterior part toward the cathode. Some remain indifferent. The reaction is usually held only a short time, and fatigue seems to occur quickly.

The behavior of this species of *Hydra* in the electric current is thus in accord with the theory and with the susceptibility gradients reported for it by Child and Hyman in 1919. The contrary behavior of the green *Hydra* may result from the fact that photosynthesis is

an endothermic reaction, while oxidations which seem to be the cause of inherent electric differences in animals are exothermic.

II. NON-FISSIONING PLANARIANS

It was previously reported (Hyman and Bellamy, 1922) that fissioning planarians tend to assume a U- or W-shaped orientation in the current, with anterior and posterior ends, and often the level of the main fission plane, directed to the cathode. This orientation was independently confirmed by Mr. Bench for *Planaria dorotocephala*. He found 80 per cent of cathodic response with U- and W-shaped orientations toward the cathode. The presence of zooids in the posterior parts of fissioning species is believed to account for the characteristic posture, since the anterior end of each zooid constitutes a region of electric positivity.

It seemed to me that it would be interesting to try out species of planarians known to lack asexual reproduction. One such form is *Curtisia foremanii* (=*Planaria simplissima*). Thirteen specimens were tested separately. The voltage necessary to induce a definite response ranged from 5 to 15. Smaller specimens required a higher voltage than larger ones. The behavior of all thirteen was alike. In each case, either immediately when the current was passed or after some hesitation, the animal turned its anterior end toward the cathode and crawled toward the cathode with its body parallel to the direction of the current. There was no indication of a U- or W-shaped orientation as in fissioning species. Upon reversal of the current, the orientation was also reversed.

Another species tried was *Procotyla fluviatilis*, the common white flatworm of the northeastern United States commonly called *Dendrocoelum lacteum*. Fourteen specimens were tested. The voltage used ranged from 10 to 20. Of the fourteen specimens all were cathodic; four crawled to the cathode with their bodies in line with the current, while ten showed some tendency to the U-shaped orientation or to a slight bending of the posterior end to the side. In some the tail was turned under the body. These results indicate that there is some special region of metabolic activity in the posterior end of this species. The form is entirely incapable of fission and even of regeneration (of the head) except at anterior levels. It possesses at

all seasons of the year a highly complex sexual apparatus near the rear end but this is degenerate except from September to January (the experiments were performed in March). The rear end is especially adhesive since the animal often crawls in a leechlike manner. These various factors may account for a special region of positivity in the rear end, and so for the tendency to a U-shaped orientation. A few tentative trials of carbon-dioxide production of pieces of different levels by means of the color change of phenol red also indicated a metabolic rise in posterior levels.

Of these two species of non-fissioning planarians, one, then, behaves according to expectation, showing no evidence of secondary positive regions in the posterior part. It is cathodic, placing its body in direct line with the current. The other species, however, shows some tendency toward a U-shaped orientation, indicating a region of positivity in the posterior part. Some special conditions are mentioned which may account for this.

III. SUMMARY

According to the theory that galvanotactic orientation depends upon inherent electrical charges in the animal, *Hydra* should be cathodic. Pearl reported the green *Hydra* as anodic; but the brown *Hydra* (*Pelmatohydra oligactis*) is here reported as cathodic, in accordance with the theory.

Fissioning planarians should, according to the theory, possess a secondary region at the fission plane which is positively charged and therefore should assume a U- or W-shaped orientation in the current. This is the case as previously shown and again confirmed. Non-fissioning planarians should orient parallel to the current. This is here reported to be the case for one non-fissioning species, but a second species showed some tendency to a U-shaped orientation. This is contrary to expectations but may be accounted for by some special condition in the rear part of this species. Both are cathodic.

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THE HYDROGEN-ION CONCENTRATION OF THE ALIMENTARY TRACT OF NORMAL ALBINO RATS

(Three figures)

T. P. SUN, R. BLUMENTHAL, E. H. SLIFER, E. C. HERBER, AND C. C. WANG

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THAT the hydrogen-ion concentration of the gastric juices plays an important rôle in enzyme activity has been recognized for a long time. More recently, investigators have been correlating gastric pH and the utilization of various chemical elements in rachitogenic diets (Yoder, 1927; Abrahamson and Miller, 1925; Grayzel and Miller, 1928). These latter workers list numerous papers devoted to work along similar lines.

The wide use of rats in such feeding experiments makes it essential that complete data be obtained of the pH of the alimentary tract of normal rats. Investigators have been more interested in the abnormal condition, so that the number of control rats used has usually been too few to show definitely just what the normal condition may be. Only one paper on the pH of the alimentary tract of normal rats has been found, that of Redman, Willimott, and Wokes (1927). However, the number of rats which these authors used, although not mentioned, appears to be small. A recent paper by McLaughlin (1931) on the hydrogen-ion concentration of the alimentary tracts of fowl, cat, and rabbit is more suggestive than conclusive.

The present experiments were begun under the direction of Drs. J. H. Bodine and H. H. Donaldson, to whom our appreciation is extended for their interest and helpful criticism. Thanks are also due to the Wistar Institute, which so kindly supplied us with rats from their experimental strain colony.

MATERIALS AND METHODS

Two hundred and fifty male and female albino rats, varying in age from 15 days to 2 years were used. No attempt was made to keep

the rats on any specific diet except for the regular rotary diet as used by Wistar, since the purpose of these experiments was to use animals which were normal in every way possible. Redman, Willimott, and Wokes (1927) report that varying the protein/fat/carbohydrate ratios of normal diets produced no marked alteration in the intestinal pH of adults rats. The same has been found true for dogs by Grayzel and Miller (1928).

The rats were killed by a blow on the head at 4–5 hours after feeding time. The alimentary tract was immediately removed; and the contents of the stomach, small intestine, caecum, and large intestine washed into separate centrifuge tubes with distilled water. These were covered with paraffin oil and centrifuged. Hydrogen-ion determinations were made electrometrically by means of a Clarke hydrogen electrode or a hydroquinone electrode.

The hydrogen electrode was used exclusively on 100 rats in 1928. The following year, one of us extended these experiments, using the hydroquinone electrode and 150 rats. Both electrodes were checked against buffers of known pH before and after each day's experiments. In the earlier series of experiments, Dr. D. H. Wenrich made a study of the protozoan fauna as found in the caeca and small intestines.

RESULTS

The results obtained with the hydrogen electrode are shown graphically in Figure 1. All points on a single vertical axis represent the data obtained from the same rat. The protozoan distribution in the small intestine and caecum has been plotted in units from 1 to 4, 1 being a slight infection while 4 means a heavy infection. In Figure 2 are shown the individual readings obtained using 150 rats and the hydroquinone electrode.

Analyses of these two graphs show a fair degree of variability between rats of the same age. The greatest fluctuation occurred in the stomach, whereas the small intestine exhibited a remarkable constancy. This latter condition becomes significant when we realize that most of the enzyme activity occurs in the small intestine. No significant differences in the pH of the alimentary tract between male and female rats are evident. Age differences also seem negligible except in the stomach, where the pH of young rats seem to be

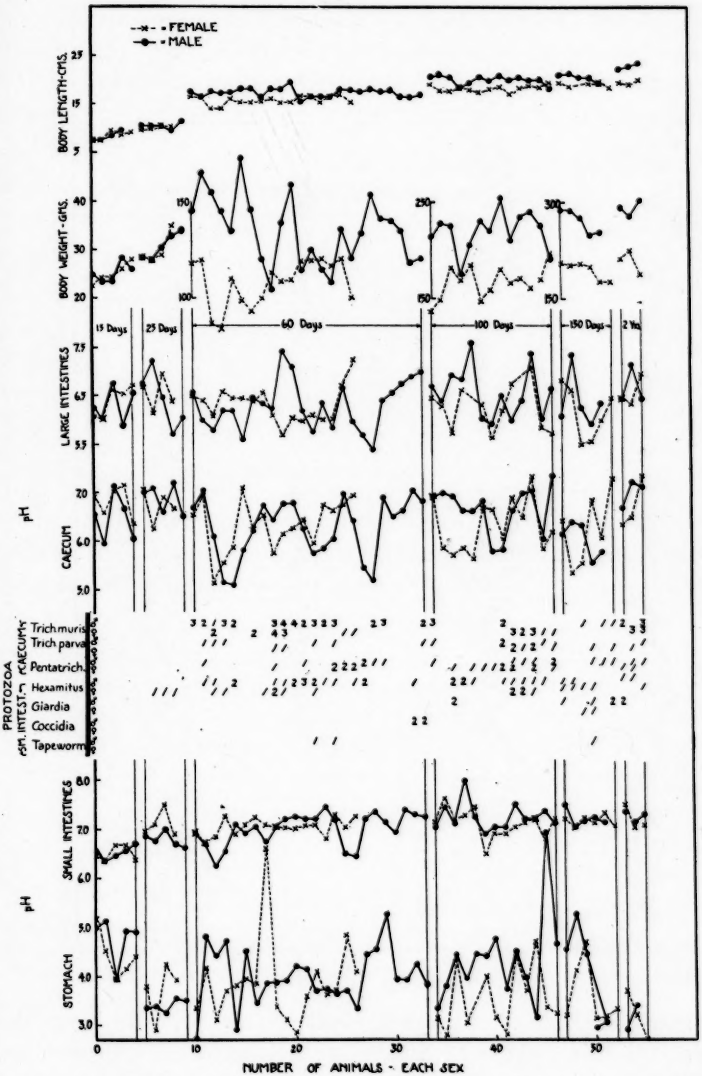

FIG. 1.—Hydrogen-ion concentration of the alimentary tract of normal rats, using the Clarke hydrogen electrode. All points along a single vertical axis represent readings from the same animal. Solid lines represent males; and dotted lines, females. The degree of protozoan infection is denoted by the numbers, 1 indicating a slight infection while 4 indicates a heavy infection. One hundred animals.

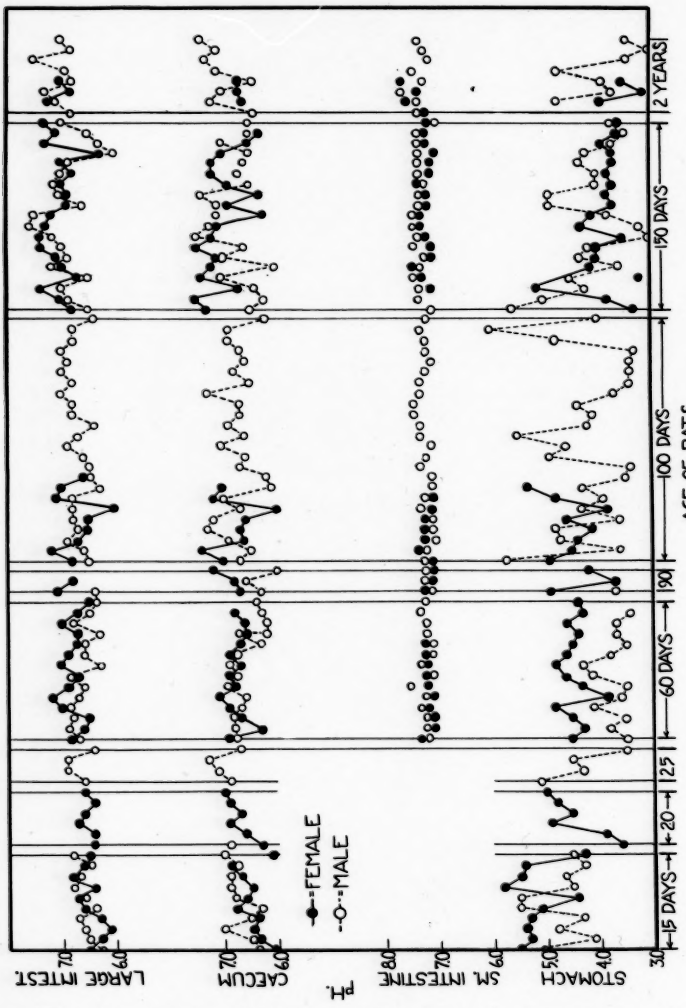

FIG. 2.—Hydrogen-ion concentration of the alimentary tract of normal rats using the hydroquinone electrode. Same as Figure 1. One hundred and fifty animals.

higher than that of older rats. However, in the 15-day rats, this high pH may possibly be due to the fact that these rats were still milk-fed.

Averages of the pH values of the various regions of the alimentary

tract have been calculated; but this was done, not with the idea of making these figures significant, but merely to make comparisons. The fluctuations in pH observed are due not so much to experimen-

TABLE I

RANGES AND AVERAGES* OF THE pH OF THE ALIMENTARY TRACT OBTAINED WITH THE CLARKE HYDROGEN ELECTRODE

AGE (DAYS)	STOMACH		SMALL INTESTINES		CAECUM		LARGE INTESTINES		NO. OF ANIMALS
	Ranges	Av.	Ranges	Av.	Ranges	Av.	Ranges	Av.	
15	4.0–5.1	4.6	6.4–6.7	6.5	6.0–7.0	6.6	6.0–7.0	6.4	10
20–25	3.4–4.0	3.5	6.6–7.1	6.9	6.3–7.1	6.8	6.2–7.1	6.5	9
60	3.4–4.7	3.9	6.5–7.2	7.0	5.5–7.3	6.3	6.0–7.0	6.3	41
90–100	3.0–4.6	3.9	6.9–7.3	7.2	5.8–7.0	6.5	5.8–6.9	6.4	26
150	3.2–4.6	3.8	7.1–7.3	7.2	5.6–6.8	6.2	5.6–6.8	6.3	11
2 years	2.9–3.6	3.2	7.1–7.4	7.2	6.4–7.3	6.9	6.4–7.0	6.6	6

* The average values of pH were obtained from the average values of C_H.

tal error as to the inherent variations which are present in the individuals themselves. Thus, a single figure to denote the pH of any region of the alimentary tract of rats of any particular age would be

TABLE II

RANGES AND AVERAGES* OF THE pH OF VARIOUS REGIONS OF THE ALIMENTARY TRACT OBTAINED WITH THE HYDROQUINONE ELECTRODE

AGE (DAYS)	STOMACH		SMALL INTESTINES		CAECUM		LARGE INTESTINES		NO. OF ANIMALS
	Ranges	Av.	Ranges	Av.	Ranges	Av	Ranges	Av.	
15	4.3–5.5	5.0	.....	....	6.3–7.0	6.6	6.3–6.9	6.6	21
20–25	3.9–5.0	4.4	.....	....	6.6–7.0	6.8	6.4–6.9	6.6	10
60	3.5–4.8	4.2	7.1–7.3	7.2	6.2–6.9	6.7	6.4–7.0	6.7	28
90–100	3.4–5.5	4.5	7.1–7.4	7.2	6.2–7.3	6.7	6.4–7.0	6.7	39
150	3.2–4.9	4.2	7.1–7.4	7.2	6.2–7.5	6.8	6.3–7.4	6.9	41
2 years	3.1–4.5	3.7	7.1–7.6	7.3	6.5–7.3	7.0	6.8–7.4	7.0	10

* The average values of pH were obtained from the average values of C_H.

misleading. A more accurate denotation is the ranges of pH values found in the various regions. Such ranges are given in Table I for the hydrogen electrode and in Table II for the hydroquinone electrode. Figure 3 shows the averages obtained from the pH readings.

On the whole, the hydroquinone electrode gave higher values than the hydrogen electrode, although the tendencies of both are in the same direction. The effect of dilution of the samples on pH is ruled

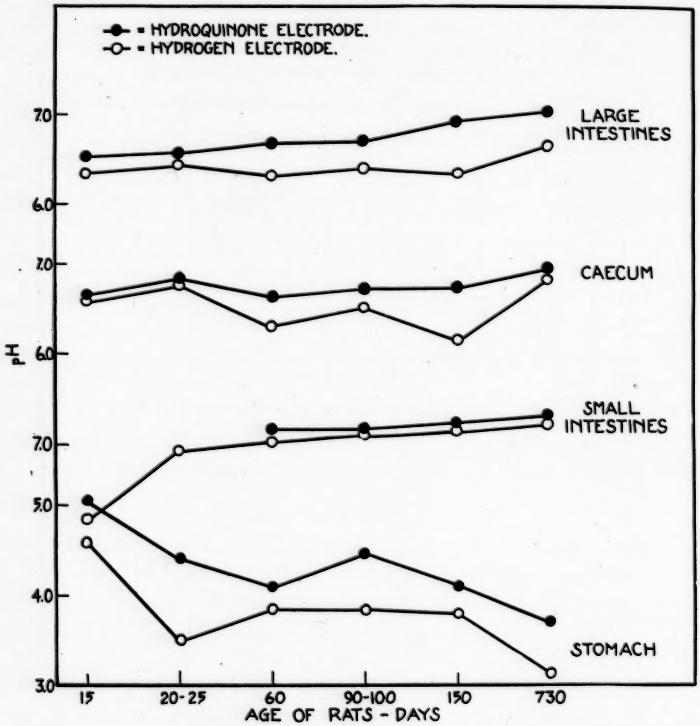

FIG. 3.—Averages of pH of the alimentary tract of normal rats, showing that the hydroquinone electrode gave consistently higher readings than the Clarke hydrogen electrode. These average values are not significant in themselves, the ranges as given in Tables I and II being of more importance.

out by the observation of Davidsohn (1913) and Grayzel and Miller (1928), who report no material alteration in pH with dilution.

SUMMARY AND CONCLUSIONS

1. The ranges of pH values in the stomach, small intestine, caecum and large intestine of 250 normal rats varying in age from

15 days to 2 years have been determined by means of the Clarke hydrogen electrode and the hydroquinone electrode.

2. The contents of the stomach showed the greatest fluctuation, while the small intestine exhibited a marked constancy in pH.

3. Except in the stomach, the alimentary tract exhibits no change in pH with change in age. In the stomach, the pH seems to fall with increase in age.

4. No significant differences were found between male and female rats.

5. The hydroquinone electrode gave consistently higher results than the hydrogen electrode.

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VARIATIONS IN THE GROWTH-PROMOTING POWER OF KIDNEY FOR PLANARIAN WORMS

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IT HAS already been shown that tissues which do not present marked differentiations macroscopically may, nevertheless, manifest very great differences in growth-promoting power for planarian worms (Bahrs, 1929*a*, 1931). The present paper is concerned with a demonstration of the differences in growth-promoting power of the cortex, boundary, and papillary zones of the medulla in the kidney of the guinea pig, rabbit, and sheep.

Through the use of the planarian worm as a test animal, one can examine very small organs for growth-promoting power. A half gram of tissue furnishes an excess of food for an experimental group of 30 worms, and this needs be provided only once or twice per week.

Our method of experimentation is the following: Only newly regenerated or young worms[2] are used. These are obtained by cutting the posterior ends from the large stock worms and allowing these posterior ends to regenerate into new individuals, the time required for this being about 3 weeks. We use worms 6, 7, and 8 mm. in length; and each experimental group consists of 30 measured individuals. The experimental groups are kept in finger bowls in sterilized river water.

The dark red cortex and the very pale papillary zone of the kidney present a striking contrast in appearance. In the first experiment a careful separation of these two parts of the guinea-pig kidney was made. The two experimental groups of worms were fed once per week over a period of 8 weeks upon these separated parts of the kidney from freshly killed guinea pigs. Each experimental group of worms had a total length of 180 mm. at the beginning of the experi-

[1] Aided by a grant from the research fund of the University of Oregon.

[2] According to Dr. Libbie Hyman, of the University of Chicago, the species used is probably *Planaria agilis*. The lack of sexually mature individuals makes identification difficult.

ment. At the end of the eighth week the worms fed kidney cortex had increased their length 78 per cent, while the worms fed the papillary zone had increased their length by but 30 per cent, the worms fed cortex thus making a gain 2.6 times as great as those fed papillary zone.

In the next experiment these two regions of the kidney of the rabbit were examined in the same way with the following results: Worms fed kidney cortex showed an original total length of 197 mm. and a gain of 100 per cent at the end of 4 weeks, while the experimental group of worms receiving the papillary zone of the medulla were 190 mm. in total length originally and gained 59 per cent. There is no doubt of the superiority of cortex of the rabbit kidney over the papillary zone of the medulla in promoting growth in planarian worms. The kidneys were from freshly killed rabbits, and the experimental groups of worms were fed twice per week.

The kidney of the sheep was next used. That sheep-kidney cortex possesses a high growth-promoting power for planarian worms has already been demonstrated (Wulzen, 1923, 1926). The sheep kidneys were purchased at the local market and had been refrigerated for an unknown length of time, which has a deleterious effect upon the growth-promoting power of some animal tissues (Bahrs, 1929*b*). Still the marked contrast in growth-promoting power between the cortex and papillary zone of the medulla was in evidence. The experimental group of planarian worms fed sheep-kidney cortex had an original total length of 195 mm. and showed a gain of 108 per cent in 4 weeks, while the group fed the papillary zone of the medulla had an original total length of 192 mm. and gained 39 per cent. Sheep-kidney cortex thus has a growth-promoting power for planarian worms 2.7 times as great as that of the papillary zone of the medulla.

Between the cortex and papillary zones of the kidney is a very narrow but distinctly marked boundary zone. In a kidney as large as that of the rabbit this boundary zone can easily be separated and tested for growth-promoting power, as is shown by the following experiment. The group of worms fed rabbit-kidney cortex had an original total length of 185 mm. and at the end of 3 weeks had gained 57 per cent. The experimental group fed the boundary zone of the medulla had an original total length of 183 mm. and showed a gain

in 3 weeks of 29 per cent, while the group fed the papillary zone, having an original total length of 181 mm., showed a gain in 3 weeks of 16 per cent. The boundary zone of the kidney is thus intermediate in growth-promoting power, when compared with the cortex and papillary zone. In 4 weeks the gain percentages were the following for the three experimental groups: cortex 96 per cent, boundary zone of the medulla 51 per cent, papillary zone of the medulla 19 per cent.

The differences in the growth-promoting power of these three regions of the kidney cannot be accounted for on the basis of food consumption, because all three were attacked with equal vigor on the part of the worms, and their bulged appearances, after having eaten, left no doubt as to their success with the food. It occurred to us that an accumulation of urinary products in the medulla of the kidney might account for the low growth-promoting power of that part, and the following series of experiments were designed to test this hypothesis.

Experimental groups of 30 worms were fed for periods of 3 or 4 weeks upon rabbit kidneys which had been perfused through the renal artery with Ringer-Locke[1] solution. In the first experiment, one kidney was perfused for approximately 1 hour at room temperature; the other kidney was used as a control. Two experimental groups of worms were fed the cortex and papillary zone, respectively, from the non-perfused kidney. Two similar groups were fed the cortex and papillary zone, respectively, from the kidney which had been perfused by way of the renal artery with Ringer-Locke solution for approximately 1 hour. About 50 cc. of solution passed through the kidney in this time, with the result that it had a very "washed-out" appearance and had swollen about one-third beyond its original size. The results, as given in Table I, show that perfusion had a consistent, deleterious effect upon the growth-promoting power of these two regions of the rabbit kidney.

In the next experiment the Ringer-Locke solution was made more alkaline, pH 8.5, by the addition of N/10 NaOH, in the hope of increasing the permeability of the cells in the kidney and perhaps washing out more of the growth-promoting substances. Perfusion

[1] NaCl, 9 gm.; KCl, 0.42 gm.; $CaCl_2$, 0.24 gm.; $NaHCO_3$, 0.1 gm.; glucose, 2.0 gm.; H_2O, to 1,000 cc.

for 1½ hours was carried on in an incubator at 35–38° C., during which time about 50 cc. of solution passed through the kidney. As in the first experiment, one kidney served as a control while the other

TABLE I

EFFECT UPON THE GROWTH-PROMOTING POWER OF PERFUSING KIDNEY WITH RINGER-LOCKE SOLUTION; ROOM TEMPERATURE; TIME APPROXIMATELY 1 HOUR

Food Fed Worms	Original Total Length of 30 Worms (Millimeters)	Gain in Total Length in 3 Weeks (Millimeters)	Percentage of Gain in 3 Weeks	Gain in Total Length in 4 Weeks (Millimeters)	Percentage of Gain in 4 Weeks
Cortex, unperfused	216	133	61	168	77
Cortex, perfused	216	106	49	107	49
Papillary zone, unperfused	216	47	21	97	44
Papillary zone, perfused	216	41	18	54	25

was perfused. The results are given in Table II, and show that all three regions of the kidney lost in growth-promoting power through perfusion with Ringer-Locke solution at pH. 8.5. However, the in-

TABLE II

SHOWING THE EFFECT UPON THE GROWTH-PROMOTING POWER OF PERFUSING KIDNEY FOR 1.5 HOURS WITH RINGER-LOCKE SOLUTION AT pH 8.5

Food Fed Worms	Original Total Length of 30 Worms (Millimeters)	Gain in Total Length in 3 Weeks (Millimeters)	Percentage of Gain in 3 Weeks	Gain in Total Length in 4 Weeks (Millimeters)	Percentage of Gain in 4 Weeks
Cortex, unperfused	185	106	57	178	96
Cortex, perfused	181	69	38	154	85
Boundary zone of medulla, unperfused	183	54	29	94	51
Boundary zone of medulla, perfused	180	43	23	67	37
Papillary zone of medulla, unperfused	181	30	16	35	19
Papillary zone of medulla, perfused	180	6	3	5	2

crease in alkalinity, from pH 7.3 to pH 8.5 does not appear to have been effective in increasing the removal of the growth-promoting substances, except in the papillary zone.

Thinking that a change in the channel of perfusion might modify the results, three experimental groups of worms received the three regions (cortex, boundary, and papillary zones, respectively) from

kidneys which had been perfused with the renal vein ligated, while three similar groups received these three regions from kidneys perfused with the ureter ligated. The experiment was of 3 weeks' duration, and the worms were fed twice per week upon the kidneys from freshly killed rabbits. The results, as set forth in Table III, show that perfusion with the ureter ligated was more effective in washing out the growth-promoting substances than perfusion with the renal vein ligated. This fact finds a simple explanation in the amount of solution which passed through the kidneys treated in these two different ways. An average of 9.6 cc. of solution passed through the

TABLE III

SHOWING THE EFFECT UPON THE GROWTH-PROMOTING POWER FOR PLANARIAN WORMS, OF PERFUSING RABBIT KIDNEYS WITH EITHER THE RENAL VEIN OR THE URETER LIGATED; RINGER-LOCKE SOLUTION; TIME OF PERFUSION, 1 HOUR.

Food Fed Worms	Original Total Length of 30 Worms (Millimeters)	Gain in Total Length in 3 Weeks (Millimeters)	Percentage of Gain in Total Length in 3 Weeks
Cortex, vein ligated	201	101	50
Cortex, ureter ligated	201	85	42
Boundary zone, vein ligated	201	66	32
Boundary zone, ureter ligated	201	49	24
Papillary zone, vein ligated	201	38	18
Papillary zone, ureter ligated	201	19	9

kidneys perfused with the renal vein ligated, while an average of 20.4 cc. passed through the kidneys with the ureter ligated. This experiment also demonstrates the fact that the oedema of the kidney due to perfusion is not the cause of its loss in growth-promoting power, for both kidneys were equally enlarged; and still there was a distinctly greater loss in the growth-promoting power of the kidneys through which the greater volume of solution passed.

In the following experiment, designed to show the effect of prolonged perfusion, one kidney was kept as a control while the other was perfused for 9 or $9\frac{1}{2}$ hours with Ringer-Locke solution at 35–38° C., pH. 7.3. The experiment was of 3 weeks' duration, and Table IV gives the experimental data. This experiment shows that long-continued perfusion completely removed all of the growth-promoting substances from both boundary and papillary zones of the medulla. The experimental group of worms receiving the boundary zone of the medulla lost 4 per cent of their original total length,

while the group fed the papillary zone lost 3.7 per cent. (This loss, however, is not as great as if they had received no food.) The fact that the unperfused boundary and papillary zones of the kidney did not give as good growth in this experiment as in some of the others may be accounted for in this way: The worms were fed twice per week; but rabbits were killed once per week, and part of the kidney was held over in a frozen condition until the next feeding. Refrigeration does have a deleterious effect upon certain animal tissues (Bahrs, 1929*b*). However, both perfused and non-perfused kidneys were subjected to refrigeration.

TABLE IV

SHOWING THE EFFECT OF 9 HOURS' PERFUSION WITH RINGER-LOCKE SOLUTION UPON THE GROWTH-PROMOTING POWER OF RABBIT KIDNEY FOR PLANARIAN WORMS

Food Fed Worms	Original Total Length of 30 Worms (Millimeters)	Gain in Total Length in 3 Weeks (Millimeters)	Percentage of Gain in Total Length in 3 Weeks
Boundary zone, unperfused	215	37	17
Boundary zone, perfused	213	− 9	− 4
Papillary zone, unperfused	212	3	1.4
Papillary zone, perfused	212	− 8	− 3.7

It will be observed that results on kidney cortex do not appear in this last-reported experiment (Table IV). It frequently happens that planarian worms develop a peculiar malady when fed on kidney cortex, and the reason for this we do not know. Their outlines become irregular, and in severe cases they curl up and are unable to move. They continue to eat, but of course cannot be measured in this condition. In previous experiments (unpublished) we found that a diet of refrigerated kidney tissue was especially effective in bringing on this malady in the worms. In the last-reported experiment (Table IV) three experimental groups were fed upon kidney cortex, and practically all of these worms developed this malady. The condition affects only the worms fed kidney cortex, for those fed upon the boundary and papillary zones of the medulla have never been known to develop this disease.

These experiments show that an accumulation of urinary products in the medulla of the kidney cannot account for its low growth-pro-

moting power, for perfusion of the kidney lowers the growth-promoting power of all parts. The worms gathered upon and consumed the perfused tissue as vigorously as they did the non-perfused kidney, and in all cases there was no doubt but that food consumption was adequate. Therefore a consideration of the histological structure of the kidney may furnish some hint as to the reason for these differences in the growth-promoting power in the three regions of the kidney. Whether the tissue was ground to a fine paste or given as a whole piece made no difference in the comparative results, and one would therefore expect to find some cellular difference responsible for the variations in growth-promoting power.

All parts of the kidney are made up of the uriniferous tubules. In the cortex we find the Bowman's capsules, the proximal and distal convoluted tubules, and the beginnings of the straight collecting tubules. The boundary zone consists of the ascending and descending limbs of Henle's loop and the central portions of the straight collecting tubules. The papillary zone consists of the posterior portions of the straight collecting tubules and also the wide ducts of Bellini, into which enter numbers of straight collecting tubules.

The whole uriniferous tubule from beginning to end is lined with epithelium, but the character of the epithelium differs greatly in different parts of the tubule. The epithelium of the Bowman's capsule is flattened, and the cytoplasm of the cells is either clear or finely granular. The first, or distal, convoluted tubule and the spiral tubule which joins it to the descending limb of Henle's loop have a very thick epithelial lining. The cytoplasm of these cells contains abundant mitochondria, and these have a tendency to be arranged in longitudinal rows, as in the cells lining the salivary glands. This striated appearance of the mitochondria has given these cells the term "rodded epithelium." Granular mitochondria are also abundant. The cells also frequently exhibit a brush of cilia-like processes projecting into the lumen, but these are not vibratile. In the zigzag tubule which joins the ascending limb of Henle's loop and the proximal or second convoluted tubule, and in the convoluted tubule itself, "rodded epithelium" is also present. Bowman's capsule, the distal and proximal convoluted tubules, and the spiral and zigzag tubules are all in the cortex of the kidney. In the boundary zone of the medulla are the descending and ascending limbs of Henle's loop

and a portion of the straight collecting tubule. The epithelium of the descending limb of Henle's loop is composed of flattened cells with clear cytoplasm, mitochondria being absent or rare. However, in the ascending limb of Henle's loop rod-shaped or granular mitochondria are always present, and the cells are cuboidal in shape. The papillary zone of the medulla contains the ducts of Bellini and the cytoplasm of these tall columnar cells is very clear and free from granules. In the entire collecting tubule, from its beginning in the cortex of the kidney to its end in the papillary zone of the medulla, the clear cytoplasm and distinct nuclear membranes stand out in striking contrast to the granular cytoplasm in the secreting portions of the uriniferous tubule.

In the cortex of the kidney there is much of the "rodded epithelium" and an abundance of other types of mitochondria, and the growth-promoting power for planarian worms is high. The boundary zone of the medulla has very much less of mitochondria-containing epithelium, for only the ascending limb of Henle's loop has cells containing mitochondria; and the growth-promoting power is very much lower. The cytoplasm of the straight collecting tubules is devoid of granules of any kind, and the papillary zone of the kidney contains only collecting tubules. The growth-promoting power of the papillary zone of the medulla is very low, indeed, when compared to that of the cortex of the kidney. Therefore, the high growth-promoting power of the cortex of the kidney appears to be associated with the presence of abundant mitochondria granules in the cytoplasm of the cells. There are, of course, the glomeruli in the cortex; but if glomeruli were the factor concerned, the growth-promoting power of the boundary zone of the medulla should be as low as that of the papillary zone, since the boundary zone of the medulla contains no glomeruli. On the contrary, the boundary zone falls between the extremes of the cortex and papillary zone of the medulla, which is where it would be placed if abundance of mitochondria were a factor in high growth-promoting power for planarian worms. There is the possibility that the presence of mitochondria is simply the outward sign of a deep metabolic difference between the cells which contain them and the cells which do not and that it is upon this more fundamental phenomenon that growth-promoting power depends.

SUMMARY

1. An analysis of the cortex and medulla of kidneys from the guinea pig, rabbit and sheep shows that the growth-promoting power of the cortex far exceeds that of the medulla for planarian worms.

2. The medulla of the kidney is divided into two parts, the boundary and papillary zones. A separation of these two regions was made in rabbit kidneys, and it was found that the growth-promoting power of the boundary zone is considerably greater than that of the papillary zone.

3. High growth-promoting power in the kidney appears to be associated with the presence of an abundance of mitochondria in the cytoplasm of the epithelial cells, for the cortex has an abundance of mitochondria, the boundary zone very much fewer, and the papillary zone has none.

4. The growth-promoting power of rabbit kidney, for planarian worms, is decreased by perfusion with Ringer-Locke solution, the loss in growth-promoting power being proportional to the length of time of perfusion.

5. Perfusion for 9 hours so removes the growth-promoting substances from kidney tissue that it has less than a maintenance value.

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SPEED OF TOXIC ACTION OF MERCURIC CHLORIDE ON DAPHNIDS

(Three figures)

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THE killing rate of a poison generally increases as a more concentrated solution is used, whether the poison be fed or injected, or whether the animal be placed directly into the poison solution. The relationships involved are varied. Sometimes there is a simple linear relation, but usually the effects are more complicated.

Relations of more or less regularity were found by Chick (1908), Collett (1919), Irwin (1925), Brinley and Baker (1927), Carpenter (1927), Shackell (1925), Hartmann (1918), Cook (1926), Powers (1920), and others.

The process of killing by toxic action is, in common with most other biological processes, speeded up by an increase in temperature; but there are great quantitative variations in the relative amounts of the increases.

Several have reported a linear relation between the logarithm of the rate of action and the reciprocal of the absolute temperature; that is, the reaction responds to temperature changes according to the law of van't Hoff. Over a short range this is practically equivalent to the Arrhenius-Marcelin-Rice equation, so far as fitting biological data is concerned. Such increases are reported by Carpenter (1927), Hartmann (1918), Powers (1920), Sorbi (1921), Cameron and Hollenberg (1922), and others. Observers reporting other more or less regular temperature responses are Sollman, Mendenhall, and Stingel (1914), Collett (1919), Irwin (1925), and Brinley and Baker (1927).

The present paper presents data concerning the effects of changes in concentration and temperature on the rate of killing of daphnids by mercuric chloride.

It is a pleasure to acknowledge indebtedness to Dr. L. A. Brown for suggestion of the problem and for guidance throughout its completion, and to Dr. J. H. Bodine for many helpful suggestions.

MATERIALS AND METHODS

The animals used were adult, parthenogenetic females of *Simocephalus vetulus*, collected from Lake Okoboji in northwestern Iowa. They were killed by being placed directly into the poison solution. For each individual the killing time was noted with a stop watch. The time taken for the death-point was the last observable movement, which was almost invariably the extension of the postabdomen. Each collection of animals was divided into several samples and killed at various temperatures and concentrations, so as to distribute variations. The number in any one series was usually about 25 or 50, and the smallest number for any one concentration at any one temperature was 100.

The concentrations of mercuric chloride used were from 0.1 N to 0.0001 N. Beyond these limits various errors and difficulties enter in, so that higher and lower concentrations were not practicable. The distilled water from which the solutions were made was found to cause death in a few hours, but this may be due to traces of harmful substances in the water and not to the distilled water itself. Preliminary observations had shown that there are precipitations and pH changes in the mercuric chloride solutions (especially the dilute solutions) when they stand for several days; to minimize the effects of such changes, fresh solutions were made up for each series of experiments.

The temperature range was from 10° C. to 30° C. Within this range there are probably no abnormal effects due to the temperature itself. Brown (1926) found that this species behaves normally with respect to instar length at least up to 34°, and MacArthur and Baillie (1929) found that the effects of low temperatures on *Daphnia magna*, very similar to *Simocephalus* in its temperature relations, begin at about 6°. When animals were killed at temperatures very different from that of the water from which they were collected, they were allowed to stand in the lake water so as to come to the desired

temperature very gradually. They were usually killed within a few hours after they were collected, and in no case later than 16 hours after collection.

RESULTS

When the number or percentage of animals killed in successive time-intervals is plotted against the corresponding time-intervals, a sigmoid killing curve is obtained. For each concentration there is a series of such curves, all roughly the same shape, the corresponding times being longer as the temperatures are lower. A typical series of such curves, those for 0.01 N mercuric chloride, is shown in Figure 1.

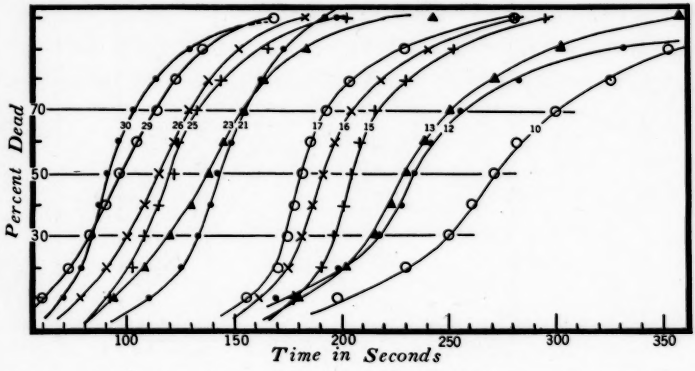

FIG. 1.—Series of killing curves of 0.01 N mercuric chloride at various temperatures from 10° to 30° C. The individual killing times are arranged in deciles for plotting the points.

The effect of concentration changes may be shown by plotting any set of corresponding points in the killing curves, or the mean killing times as calculated from the data, against the concentrations used, the temperature being constant. For convenience in later use of the graph the killing rate, obtained by multiplying the reciprocal of the killing time by 10^4, is used instead of the time, and the logarithm of the reciprocal of the concentration is used instead of the concentration. This decreases the range of the numerical values and gives positive figures for plotting. The resulting graphs (log rate against log reciprocal concentration) are linear, being pairs of

straight lines intersecting at a point between 0.001 N and 0.0031 N. Figure 2 shows these relations, the indicated points being the rates as determined by the times for 50 per cent dead and by the mean killing times.

Straight-line relations are also evident when the logarithm of the rate (rate determined as above) is plotted against the reciprocal of

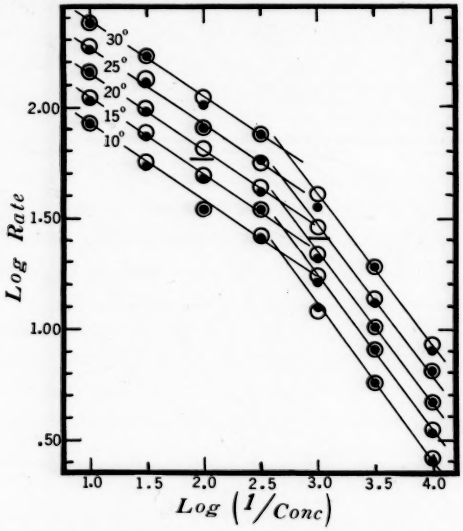

FIG. 2.—Relation between killing rate and concentration. The rate is the reciprocal of the killing time; the concentration is in terms of normality. The open circles represent the rate for 50 per cent dead; the closed circles, as determined by the calculated mean killing time. The two underlined points were determined by interpolation from the temperature data.

the absolute temperature. The slope of the lines is roughly the same for all the concentrations used, and the lines are about parallel for the different methods of computing the killing rate. Figure 3 shows the temperature relations.

DISCUSSION

The curves of Figure 2 show that the rate of killing falls with a decrease in concentration, but that the relative value of the decrease is greater for the lower concentrations. Such breaks were obtained

by Campbell (1926) and Powers (1921) in somewhat similar studies, but under different conditions. A break of this sort may be due to a differential response on the part of the organism or to a difference in the poison. Mercuric chloride, being the salt of a strong acid and a weak base, is hydrolyzed to a considerable extent, but just how is

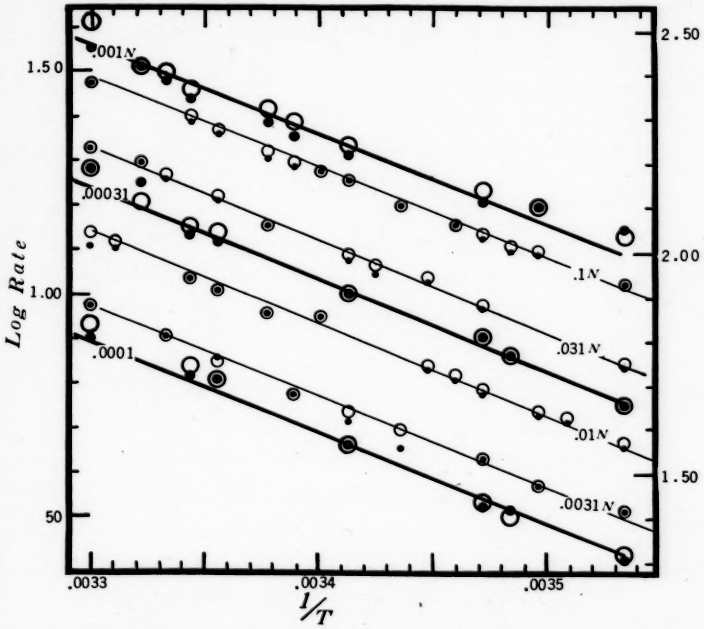

FIG. 3.—Relation between killing rate and temperature. Points determined as in Figure 2. Lines drawn according to equations (3) and (4) on page 213. Graphs for the four higher concentrations (light lines and small circles) drawn to the right-hand scale; those for the three lower concentrations (heavy lines and large circles) to the left-hand scale. Scales from log rate 0.40 to 1.65 and from 1.30 to 2.50 superimposed to decrease the vertical range of the figure.

not known. The effect of hydrolysis on toxicity depends on two factors: the lessened effective concentration of the salt itself, and the relative toxicity of the hydrolysis products. If the latter are only slightly toxic, or if they exert an influence antagonistic to that of the original salt, the killing time will be much longer than would be the

case without hydrolysis. There may also be a difference between the type of response to solutions above and below a critical concentration with a threshold value at which the other type of response sets in.

A heavy metal salt may act by forming powerful adsorption complexes at the surface or by penetration. In either case the effects are probably due to differential effects upon the velocity constants of a number of normally consecutive reactions. According to Osterhout (1917), Mellor (1914), Cook (1926*b*), and others, there is a system of such reactions, as $O \rightarrow A \rightarrow M \rightarrow B$, where O is the original substance and B the end-product and M an intermediate substance determining some normal condition of the cell, such as permeability. Interfering toxins might stop or slow down the reaction $O \rightarrow A$; and then as $A \rightarrow M \rightarrow B$ goes on, the supply of M is exhausted and death ensues. The change in the amount of M resembles a monomolecular reaction, and it is the velocity constant of this apparent reaction that shows up in the killing curves. This constant will depend on the amount of heavy metal present in the system in effective form.

The concentration effects may be expressed by the equation:

$$\log S = \log K - k\left(\log \frac{1}{C}\right), \quad \text{or} \quad S = KC^k, \tag{1}$$

where S is the killing rate, C the concentration, K and k constants. Log K, then, represents the distance from the origin to the point of intersection of the concentration curve and the zero ordinate, and k the slope of the concentration curve, or

$$\frac{\log S_2 - \log S_1}{\log \frac{1}{C_2} - \log \frac{1}{C_1}}.$$

The effect of temperature may be expressed by the symbols μ and Q_{10}. The former is the thermal increment of the Arrhenius-Marcelin-Rice equation:

$$2.31 \log \frac{S_2}{S_1} = \frac{\mu}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right).$$

The latter is the rate of action at one temperature divided by the rate at another temperature 10° lower. If the Arrhenius equation is written for a 10° interval, it may be expressed:

$$\frac{K_1}{K_2} = e^{R\left(\frac{10}{T_1 T_2}\right)} .$$

The right-hand term of this equation is equivalent to Q_{10}, since it represents the increase in rate of action per 10° rise in temperature.

The temperature data may also be put into the form of an equation resembling the concentration equation:

$$2.31 \log S = K - k \cdot \frac{1}{T}, \qquad \text{or} \qquad S = e^{K - k \cdot \frac{1}{T}}. \tag{2}$$

Here k is the slope of the temperature curve, and since μ is the slope of the same line, μ equals k times R. Hence this equation becomes:

$$2.31 \log S = K - \frac{\mu}{RT}.$$

Equations (1) and (2) may be combined into one of the following forms:

$$2.31 \log S = K - a \log \frac{1}{C} - b \frac{1}{T},$$

or

$$2.31 \log S = K - a \log \frac{1}{C} - \frac{\mu}{RT}.$$

Fair agreement is secured when the following values are used, μ being 9,500. For the concentrations from 0.1 N to 0.0031 N,

$$2.31 \log S = 9.49 - 0.34 \log \frac{1}{C} - \frac{\mu}{RT}. \tag{3}$$

For the concentrations from 0.001 N to 0.0001 N,

$$2.31 \log S = 10.38 - 0.68 \log \frac{1}{C} - \frac{\mu}{RT}. \tag{4}$$

Figure 3 shows the lines plotted according to equations (3) and (4) and the points placed according to the observed data. The values of μ, as determined by the graphs of the individual experiments, ranged from 9,100 to 10,100, with an average value of 9,500. The Q_{10} values, taken directly from the numerical data, range from 1.59 to 1.88, with an average of 1.75.

The value 9,500 for μ is neither a common nor a rare one for biological processes. In the multimodal frequency polygon of temperature characteristic distribution compiled by Crozier (1926), this value lies in one of the "troughs," but the absolute frequency is greater than that of some of the peaks of the curve.

THE KILLING CURVE

A great many biological processes can be represented by comparatively simple equations, such as those of the typical chemical reactions; but the curves of biology are statistical expressions of variation, in this case variation in resistance to a toxic agent. If the distribution of the killing times is about equal on both sides of the mean, the data can be put into the form of a symmetrical sigmoid curve. If there is a larger number of variants on one side of the mean than on the other, the curve will be asymmetrical. Sigmoid curves of killing are reported by Wood (1924), Peters (1920), Boycott (1920), Shackell (1925), Cook (1926*a*), Brown and Crozier (1927), Packard (1926), and others.

If the variation is all on one side of the mode, the resultant integral curve is not sigmoid at all. Chick (1908) obtained such curves in killing bacteria, the first time-interval being the one with the greatest frequency. Even in such cases it is possible that the curves are really sigmoid, but that the first part of the curve is missed due to the rapidity of the action. Trevan (1927) points out that mortality curves are typically sigmoid, with wide variations in exact shape.

If there is more than one mode to the distribution curve, there will be more than one point of inflection to the killing curve. Fulmer and Buchanan (1923) found such curves in a series of experiments on the effect of phenol on yeast cells.

Equations assigned to such biological processes must necessarily

be purely empirical, and cannot express all the physical and chemical factors involved, but only a summation of all of them. The equation

$$X = \frac{a}{1e^{-kt}},$$

where X is the percentage dead, t the time, and a and k constants, can be made to fit with a fair degree of accuracy, although it is the equation of a symmetrical curve. The constant a must be made larger than 100 because the killing rate does not approach the 100 per cent line asymptotically, but reaches that line. The whole curve must be shifted so as to make the theoretical and the observed points of inflection coincide.

PHYSIOLOGICAL EFFECTS

The heavy metals are usually considered to be toxic because of the strong adsorption complexes that they form, causing stoppage of cell commerce by their failure to break up. The local effect generally depends on strong adsorption by albumin. According to Poulsson (1923), the albuminates formed differ from ordinary salts in not being constituted according to definite proportions, so that the amount of the metal present may vary within wide limits. The protein reached by the metal is precipitated and rendered lifeless. The acid radical set free by the precipitation of the metal may set up a secondary action of its own.

Most of the metals precipitate albumin as an insoluble compound and thus confine the action to the surface. A few produce albuminates that are of looser consistency, and some that are soluble in excess protein or excess salt. These do not protect the underlying tissue and may penetrate much deeper. Mercury is of the latter type.

Carpenter (1927) found in the case of minnows that there was actual suffocation due to a coating of albuminate on the outside of the gills. When insufficient metallic ion is present in the solution, the film is soon shed and the animal recovers. Analysis of the body residue shows not a trace of poison within the body. This may also be the method of killing in the daphnids. No analyses were made; but groups of 50, 100, 150, and 200 were killed by sealing in small

cells in very little water. Death occurred in from 12 minutes to 2 hours, with the characteristic symptoms of mercury poisoning. The behavior of daphnids also parallels that of the minnows in that a large number of animals in a very small amount of dilute solution may not be killed at all, while a few animals in a solution of the same concentration are all killed in the usual time, indicating that in a dilute solution there is not enough metal present to kill when divided among so many.

Cook (1926*b*) explains the toxic action of the heavy metals by assuming a reversible reaction which activates the toxic element and enables it to inhibit the normal oxidation catalysts. He finds that with log concentration and log speed of killing plotted on the same scale, the slopes of the lines are the same for the salts of several of the heavy metals, which suggests that the action of all of these elements is the same.

SUMMARY

The killing curve of *Simocephalus vetulus* with mercuric chloride in concentrations from 0.1 N to 0.0001 N is a sigmoid curve with the point of inflection at, or slightly before, the middle of the curve.

The speed of toxic action varies as a constant power of the concentration, the power being higher for the lower concentrations. The graph is a pair of straight lines intersecting between 0.001 N and 0.0031 N.

The effect of temperature on the speed of action is according to the Arrhenius-Marcelin-Rice equation, the values of the thermal increment being about 9,500 calories.

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SEX CONTROL IN CLADOCERA. VII. MALE PRODUCTION IN RELATION TO TEMPERATURE

(One figure)

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SINCE cladocerans live in ponds and lakes and are subject to varying temperatures, it is natural to assume that male production might be related to temperature. Several writers have given the results of experiments showing the effect of temperature on the life cycle of daphnids, and several have given particular attention to male production. The results of these earlier workers certainly do not agree in every case. Kuttner (1909), working with several species of Cladocera, concluded that temperature is not a factor in male production; and she is supported in her opinion by von Scharfenberg (1914). A common form, *Simocephalus vetulus*, was studied by Green (1919), who found no evidence from his observations that temperature is a vital factor in relation to the sex cycle, since he obtained sexual females, parthenogenetic females, and males at two widely divergent temperatures. Issakowitsch (1905), also using *Simocephalus vetulus*, found that lower temperatures call forth sexual reproduction and consequently males, and he believes that the lower temperature acts through the food supply. In a recent paper (1930) Tauson reports the results of observations of the effect of several factors of the external environment on the sex ratio of the ubiquitous *Daphnia pulex*. She concluded, on what appears to be rather meager evidence, that temperature is a factor in male production only below 15° C. McClendon (1910), working with this same species, *Daphnia pulex*, thought that the temperature effect is accomplished through the nutrition of the animals, but his results are opposed to those of Issakowitsch and Tauson, since he found that higher temperatures resulted in increased male production. Grosvenor and

Smith (1913), using the same genus of Cladocera as we have used in the present temperature studies, found that an intermediate temperature (14° C.) gave a somewhat higher proportion of males than either extreme.

The present investigation was undertaken as part of a general study of sex control in Cladocera. General laboratory observations had indicated a possible connection between temperature and the sex ratio of the parthenogenetic young. It also was known from other data (Banta and Brown, 1929*b*) that a retardation in development of the females is associated with their production of male young. We wish to present here a more complete picture of this one factor, temperature, as a sex-controlling agent in cladocerans than has been attempted by the present writers in the past (Banta and Brown, 1924, 1925).

EXPERIMENTAL

A single clone of *Moina macrocopa* (Banta's Line 1012) was used throughout all of the temperature work. Single broods of female young to serve as mothers (of parthenogenetic young) were distributed, according to the type of experiment, among several bottles of culture medium; and these bottles kept at different temperatures. For the higher temperatures, constant-temperature cabinets were used; for temperatures below room temperature, an inside basement room and an artificial cave were employed. In the latter the temperature could be reduced beyond its normal low temperature by the continual presence of large cakes of ice. In addition to the sex of the young produced by the mothers at different temperatures as shown in the following tables, the time of release of many of the mothers was obtained

In the first type of experiment an attempt was made to determine the effect of temperature on the sex ratio when the mothers were allowed to complete their first four free-living instars, i.e., from "birth" to the production of their first clutch of parthenogenetic young, at the chosen temperature. For this purpose temperatures ranging between 12° and 30° C. were used, and at each of these temperatures different degrees of crowding of the mothers were employed. Table I gives, in summary form, the results of this series of experiments. The male percentages are given for each temperature and for dif-

ferent degrees of crowding of the mothers. In the majority of the cases the male percentages are based on fairly large numbers of young. In addition to the fact that male production may be induced in single-mother (or in 2-mother) bottles (i.e., by temperature alone and without any element of crowding), attention is called to two features of the data. In the first place, for each degree of crowding for which the data are complete over the entire temperature range,

TABLE I

DATA SHOWING MALE PRODUCTION WITH DIFFERENT TEMPERATURES AND DEGREES OF CROWDING

No. of Mothers per Bottle	Degrees Centigrade													
	12		14		17		21		24		27		30	
	Total Young	Percentage of ♂	Total Young	Percentage of ♂	Total Young	Percentage of ♂	Total Young	Percentage of ♂	Total Young	Percentage of ♂	Total Young	Percentage of ♂	Total Young	Percentage of ♂
1–3	6,177	13.0	9,516	18.0	1,517	4.1	11,953	2.9	6,038	2.6	4,348	3.3	820	2.1
4–6	318	17.0	4,415	25.3	2,696	15.1	3,758	21.2	4,000	6.7	832	12.4	1,143	15.0
7–9	260	5.8	1,844	32.2	3,512	33.9	2,221	13.8	1,697	15.3	289	0.0	1,511	28.1
10–12	126	0.0	1,444	59.6	1,356	28.4	4,559	17.2	1,450	0.4	132	9.1	1,411	26.6
13–15	.....	.....	526	51.1	2,159	28.6	1,028	31.2	523	0.0	.....	.....	1,085	17.9
16–18	.....	.....	.....	.....	627	34.0	694	39.8	.....	.....	.....	.....	791	18.1
1–12	6,881	12.6	17,219	24.9	9,081	22.5	22,491	10.0	13,185	5.2	5,601	4.6	4,885	20.2

the proportion of males is low at the lowest temperature, reaches a first peak at 14° and 17°, and decreases to a low level at 24° and 27°, again rising (except 1–3 mother bottles) at the highest temperature. In the second place, at certain temperatures, namely 12° and 24° (and probably 27°[1]), an increase in crowding of the mothers results in the diminution or elimination of male young, while at other temperatures the proportion of males either remains practically stationary or increases with crowding.

The last horizontal line of Table I gives the combined results for 1–12 mothers per bottle. This is a weighted average, since the actual numbers of females and males are totaled in each case. This combined treatment of the data appears justified because of the fact that the temperature not only regulates male production at any one de-

[1] The data for 27° C. are suggestive but perhaps not decisive, since the numbers of animals are not large.

gree of crowding but also controls the extent to which increased crowding of the mothers can call forth male young. The male percentages based on such totals give approximately the same result, regarding maximal and minimal points, as is indicated in each of the individual series. The 13–15 and 16–18 mother classes are not included in the summary, since they are not represented in all the temperature classes and, if included, would but accentuate a difference already indicated, as would other entries at these same greater amounts of crowding at the low points, for male production, of 12°, 24°, and 27° C. In this connection it is interesting to record that at 17° increased crowding of the mothers above 18 per bottle resulted in progressively increasing the male production to well above 60 per cent at 28–30 mothers per bottle.[1]

A graph of the average male percentages for all mothers from 1 to 12 is given in Figure 1. The percentages of males are plotted against the Centigrade temperature. This plot shows clearly the occurrence of the two minima and two maxima of male production in relation to the temperature scale. Only two points, however—the maximum in the neighborhood of 15° C. and the minimum at 25°—are actually determined by the plot; the incompleteness of the data at the lower and higher temperatures render the exact determination of the other two impossible.

A comparison of the proportion of males for each degree of crowding for the several temperatures (Table I) shows that the control of temperature over male production increases with increasing crowding of the mothers. This is indicated by the fact that the maxima for male production are higher and the minima are lower for the more heavily crowded bottles than for the less crowded ones. For example, the male percentages at 12° C. decrease with increasing crowding, while at 14° C. there is an increase, which results in a greater percentage difference between the number of males at these two temperatures as crowding is increased. The differences in male percentages between 12° and 14° are as follows: 1–3 mothers, 5.0 per cent; 4–6 mothers, 8.3 per cent; 7–9 mothers, 26.4 per cent; and

[1] The data used in Table I and in Figure 1 in our 1929*b* paper were obtained at 17° C. The rôle of temperature in modifying male production with greater crowding was not appreciated at the time that paper was prepared.

10–12 mothers, 59.6 per cent. The same tendency is also seen on comparing the male percentages at 24° and 30° (27° is not chosen because of the fewer animals involved) for the different degrees of crowding of the mothers. Here the difference in male percentages are: 1–3 mothers, −0.5 per cent; 4–6 mothers, 8.3 per cent; 7–9 mothers, 12.8 per cent; and 10–12 mothers, 26.2 per cent.

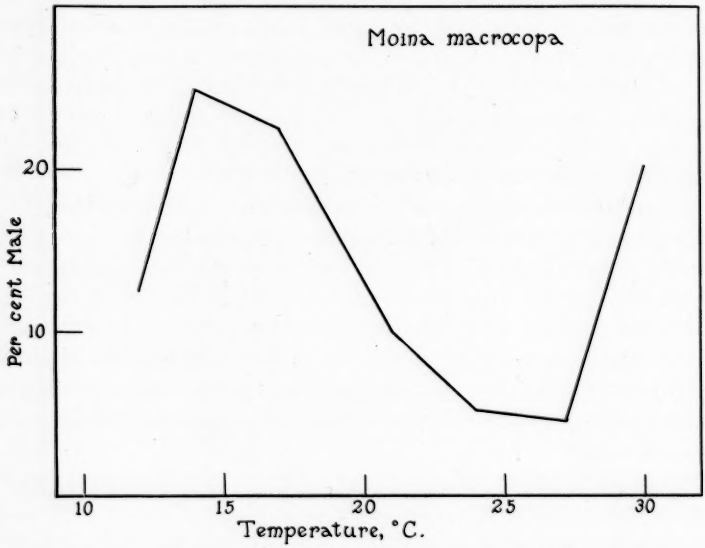

FIG. 1.—A graph of the data shown in the last horizontal line of Table I, showing the relation between male production and temperature for *Moina macrocopa*.

Since a temperature of 14° C. shows an increased male production as compared to the higher temperature range of 24°–27°, a second series of observations was made to determine the time factor involved in male causation at the lower temperature. Young females from a single brood reared at 27° were placed in bottles during their first instar in quadruplicate sets. One of these sets of bottles was left at the higher temperature as a control; the second set was placed at 14° during the first instar; a third set, transferred to this lower temperature during the second instar; and the last set was left at 27°

until the third or adolescent instar, when it, too, was placed at the lower temperature. All of the animals were left at the lower temperature until the start of the fourth instar (until the sex of the young was known to be irrevocably fixed [Banta and Brown, 1929*a*]), and then they were returned to the same temperature (27°) as the controls and the time of release and the sex of the young obtained. The summarized results of these experiments for 1, 2, 3, and 5 mothers are given in Table II. The controls yielded the usual male per-

TABLE II

DATA ILLUSTRATING RELATIONSHIP BETWEEN LENGTH OF TREATMENT AT LOW TEMPERATURE AND MALE PRODUCTION

	No. of Mothers per Bottle									
	1 Mother		2 Mothers		3 Mothers		5 Mothers		Average (1–5) Mothers	
	Total Young	Percentage of ♂	Total Young	Percentage of ♂	Total Young	Percentage of ♂	Total Young	Percentage of ♂	Total Young	Percentage of ♂
Control—at 27° C...	2,717	0.0	7,226	4.6	5,403	2.1	2,989	8.9	18,335	3.9
Third to fourth instar—at 14° C....	1,909	2.9	11,356	2.4	2,177	11.2	1,515	1.0	16,857	3.4
Second to fourth instar—at 14° C....	1,672	9.6	3,620	15.1	2,638	17.8	868	8.2	8,798	14.2
First to fourth instar —at 14° C.....	2,247	14.2	2,742	26.8	2,117	19.7	2,226	21.2	9,329	20.8

centage for this slight degree of crowding. The mothers subjected to the lower temperature during the third instar only gave substantially the same sex ratios as the controls. Lowering the temperature from the second instar until the time of egg-laying resulted in a higher male production; and a still longer exposure to the lower temperature, as in the case of the mothers reared at 14° from the first instar onward, gave an even higher male production. In this last case the females were at the lower temperature during practically all their free-living pre-adult life.

A single series of experiments is taken from the summary given in Table II and presented separately to show the type of decided response obtained in relation to temperature and the length of expo-

sure to this temperature, when other conditions are uniform. Table III illustrates this group of experiments. In this particular series no tests were made involving a transfer to the lower temperature during the second instar. Each experiment represents a single brood of

TABLE III

FURTHER DATA ILLUSTRATING RELATIONSHIP BETWEEN LENGTH OF TREATMENT AT LOW TEMPERATURE AND MALE PRODUCTION

Series 21

Exp. No.	Control, 27° C.±				Test, 14° C.							
					First to Fourth Instar				Third to Fourth Instar			
	2 Mothers		5 Mothers		2 Mothers		5 Mothers		2 Mothers		5 Mothers	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
1767	40	....	82	....	39	....	85	....	33	....	72	....
1768	41	....	80	17	22	19	71	14	45	....	84	....
1769	27	9	77	18	....	....	59	....	39	....	90	....
1770	26	6	55	18	40	....	76	1	36	....	72	....
1771	35	....	58	22	15	18	61	10	31	....	81	....
1772	33	....	63	16	31	....	49	15	36	....	80	....
					33	7						
1773	38	....	72	3	28	....	47	13	35	....	36	13
1774	31	....	78	8	15	23	34	50	33	....	75	2
1775	36	....	79	....	40	....	93	....	49	....	73	....
1776	33	....	85	1	38	....	38	56	34	....	76	....
Total number of young	340	15	729	103	301	67	613	159	371	....	739	15
Percentage of ♂	4.2		12.4		18.2		25.9		0		2.0	
Average number of young per mother	17.8		16.6		18.4		15.4		18.5		15.1	

young; and all the broods used in the several experiments were sister-broods, that is, they were granddaughters of the same individual. The average number of young per mother is given in the last line of the table and reveals an interesting result. For the same degree of crowding the number of young per mother is practically the same at both temperatures; the number is not only approximately the same but fairly large for the first broods (15–18), which indicates that all animals were living under about the same optimum

food conditions (at least until the number of eggs to be produced per mother was determined) regardless of the temperature and the degree of crowding.

DISCUSSION

It has been shown (Banta and Brown, 1929*b*) that at an intermediate temperature (17°) male production by *Moina macrocopa* is, within limits, directly proportional to the degree of crowding of the mothers. The present study reveals the fact that this simple relationship fails to hold at certain other temperatures. When a sufficiently graded series of temperatures is chosen, it is found that the proportion of males produced is characteristically different at each of the several temperatures. Two temperatures—one in the neighborhood of 14° and the other near 30°—produce maximal male production, while between these two temperatures, particularly between about 22° and 28°, the temperature effect is at a minimum.

Rather marked inconsistencies are apparent in the work of earlier writers on the effect of temperature on male production. An examination of these earlier data indicates that often but two, at most three, different temperatures were chosen, one of them usually within the range for minimal temperature effect on male production. Since this was the case, varying experimental results were obtained depending on the choice of the second (usually lower) temperature. Those who obtained practically no difference (Kuttner, von Sharfenberg, Green) probably happened to choose temperatures at points for corresponding male production either on ascending or descending limbs of a curve similar to that given here for *Moina* (Fig. 1). Not many workers have used *Moina* as experimental animals in this type of work. We would expect to find that for other species the maximal points and minimal range for male production would be shifted on the temperature scale in a manner similar to that for other vital processes in cladocerans (Brown, 1929; Brown and Crozier, 1927). The data given by Grosvenor and Smith (1913), while presenting large gaps in the temperature range, does show for the species of *Moina* used (*rectirostris*) a high male production at 14° C. and low male production at temperatures below (about 5° C.) and above

(about 28° C.) this temperature—a temperature effect obviously similar to that found for *Moina macrocopa*.

Another consideration worthy of mention is revealed by a study of the graph (Fig. 1) showing male percentages plotted against the Centigrade temperature. The shape of this graph with its initial rise to a peak, followed by a rather broad depression and a second rise, is distinctive, and recalls the type of curve obtained by Heilbrunn (1924) for the effect of temperature on the viscosity of protoplasm. If this similarity is more than accidental, then the control of sex determination in this form is intimately concerned with the physical condition of the protoplasm, male production corresponding to regions of relatively greater viscosity.

There is also a most remarkable resemblance between the graph under discussion and that given for the effect of temperature on crossing-over in *Drosophila* (Plough, 1917). Plough finds, in the case of crossing-over, that there is a first maximum at 13°, a stretch of low crossing-over percentages between 22° and 27°, and a second maximum at 31°. There is a slight drop above this last temperature. These maximal and minimal regions nearly coincide with those found for male production in *Moina*, which observation strengthens the supposition that sex determination in this form is chromosomal (Banta and Brown, 1929*a*), even though the actual chromosomal mechanism has not yet been demonstrated (Allen and Banta, 1929).

Perhaps further work with *Moina macrocopa* will extend the curve for male production and temperature a few degrees beyond the present range, but attention must be called to the upper and lower temperature limits for normal activity of this species. Different types of observations show this particular form to be extremely sensitive to lower temperatures (Brown, 1929). Ten degrees Centigrade represents the extreme limit of vigorous activity. A study of this same species at higher temperatures indicates an irregular response at temperature above 33° C. (Brown, 1926), although the true lethal temperature is much higher (Brown and Crozier, 1927).

That sex determination, whether chromosomal or otherwise, is dependent on certain antecedent changes in the protoplasm of the egg or of the mother producing eggs is indicated by the length of time that must elapse at the lower temperature before the sex ratio

of the young is altered in the male direction. Observations reported in this paper indicate that at least an instar (second instar to end of third) must have been lived at the lower temperature before any appreciable effect is noticed on the sex ratio; and the longer the mothers remain at the lower temperature (13°–14° C.) the more pronounced is the increased male production. Apparently temperature changes, if they act to alter or control the physical properties of the protoplasm which in turn predetermine the sex of the forthcoming parthenogenetic young, act gradually and cumulatively to produce a certain threshold value that must be present at the critical period (Banta and Brown, 1929*a*) for sex control near the end of the third instar.

High and low temperatures, which apparently are effective in calling forth the production of males in this particular species of daphnid, may be correlated with the sexual cycle of this form in its natural ponds. *Moina* typically are found in small pools or barnyard ponds which are subject to two hazards as a habitat for the parthenogenetic females. There is a tendency for such small ponds, since they are usually in exposed situations, to dry up in summer, the temperatures of such ponds often going well above 30° C. on bright summer days (temperatures taken at Cold Spring Harbor). A second hazard is the rapid cooling and ultimate freezing of the water as winter approaches. Under conditions conducive to either of these unfavorable situations, the sexual forms, males and sexual females, are produced and the species is protected until the return of more suitable surroundings. Field observations have indicated that males and sexual females are often found in abundance during hot summer weather.

SUMMARY

1. Single *Moina macrocopa* females, without the element of crowding, will produce male young under appropriate temperature conditions.

2. The proportion of males produced by different degrees of crowding of the mothers is characteristically different for each of several temperatures. From 14° to 21° C. male percentages appear to be increased with the crowding of the mothers, but temperatures immediately above and below this range show a maximum male

production with an intermediate number of mothers per bottle. An increase in temperature to 30° C. tends to partially restore the direct relationship between crowding and male production by the crowded mothers.

3. An analysis of the temperature effect is facilitated by grouping at a given temperature the males produced by varying degrees of crowding. When this is done, it is found that there is a peak of male production at 14°–17° C. and a second at or above 30° C. Two temperature ranges of low male production also are found (12° or below, and 22°–27° C.).

4. A possible explanation is suggested for the diverse results obtained by other workers with the effects of temperature upon male production in Cladocera.

5. The similarity of the graph for male production and temperature and the published curves for the relation between temperature and the viscosity of protoplasm suggests that perhaps male production is a function of the viscosity of the daphnid ovarian egg.

6. There is also a close approximation between the graph for male production and that for crossing-over in *Drosophila* in relation to temperature, strengthening the supposition that sex determination in this form is chromosomal in nature.

7. The effect of temperature upon the sex ratio is cumulative, in that mothers exposed to the male-inducing temperatures for a greater portion of their pre-adult lives produce more males than those exposed for a briefer period.

8. The temperature effect on the production of males is discussed in relation to the ecology of this species of cladoceran.

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THE FUNCTIONS OF THE GILLS IN BURROWING MAY FLIES (HEXAGENIA RECURVATA)[1]

(One plate)

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ALL immature May flies are thoroughly aquatic, but the burrowing ones probably remain longest in the water and have the most extensive equipments of tracheal gills. Even on slight observation, the gills of burrowers like *Hexagenia recurvata* appear to be organs of great respiratory efficiency, probably essential to life. But, the equally imposing supply of 58 tracheal gills of the caddis-fly larva *Macronema zebratum* on actual testing has appeared to be only accessory and advantageous rather than necessary to life, under ordinary conditions (Morgan and O'Neil, 1931). In view of this, these thoroughly aquatic May-fly nymphs were selected for inquiry, and the following observations were made on the functions of the gills of *Hexagenia recurvata* during the winter and early spring months.

HISTORY

The size and variety of form in the gills of May flies have attracted observation for a long time. In spite of this, few studies of their functions have been made; these are concerned both directly and indirectly with the significance of the gills in respiration. They deal with the ability of the nymphs to live without gills, and the associated question of the closed or open type of their thoracic spiracles, correlation of the gill area with the oxygen supply in the water, effects of carbon dioxide, and the influences of other physical and chemical conditions on the nymphs.

It has been shown by Cuénot (1925) and Remy (1925) that May-fly nymphs can live for 3 weeks or more without gills. In late spring (May) Cuénot removed the gills from 17 nymphs of *Cloëon dipterum*

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and 19 nymphs of *Ephemera vulgata*. One *Cloëon* nymph survived for 6 days; two *Ephemera* nymphs survived for 25 days. In late autumn (October), he took the gills from 10 more nymphs of *Cloëon*, 6 of which lived about a month and a half but were regenerating their gills within 35 days of the operation. Cuénot believed that the gill-less *Cloëon* nymphs might have secured oxygen by rising to the surface and taking air through their thoracic spiracles, which could easily have become permeable if they were not already open. He called attention to the necessity of the gills in seasons of warmth and activity and their unessential status during inactivity and in winter time (Cuénot, 1925). Commenting upon his own experiments that were similar to Cuénot's, Remy (1925) suggested that, after gill removal, nymphs might depend for at least 2 weeks upon oxygen already in their tracheae. He explained the high mortality following spring operations as due to the quicker exhaustion of the tracheal supply of oxygen. His own experiments on *Cloëon* led him to ascribe more respiratory importance to the gills than Cuénot had granted them. In experiments on gilled and gill-less nymphs briefly reported by Morgan and Porter (1929), young *Heptagenia* nymphs lived for about 5 months after gill-removal. These *Heptagenia* nymphs which were normally photo-negative became photo-positive after their gills were removed; and only 18 per cent of gill-less nymphs molted, whereas 70 per cent of the normals did so.

The question of the possible free passage of gas through the thoracic spiracles has kept reappearing. Graber (1874) reported bubbles of gas coming from the spiracles of May-fly nymphs; Heiner (1914) recorded similar bubbles escaping from thoracic spiracles of May flies when the temperature of the water was raised. No thorough study of the nymphal spiracles has yet been made, and their open or closed condition has not been actually demonstrated.

From the motion of the gills, leisurely in richly oxygenated water and vigorous where there was a poor supply, Babak and Foustka (1907) concluded them to be organs of oxygen intake. Dodds and Hisaw (1924) measured the gill area in species of May-fly nymphs living in diverse habitats, and showed that there is a definite correlation between gill area and oxygen content of the water about them. Numerous experiments were performed on normal nymphs of *Cloëon*

dipterum by Pruthi (1927) analyzing their habitats from the standpoint of respiration. His results indicated that May-fly nymphs can endure a very low concentration (1.0 cc. per liter) of oxygen, that the hydrogen-ion concentration was highly significant to them, but that the carbon-dioxide pressure was of greater importance and might be a guide to the suitability of the habitat.

MATERIAL AND METHODS

The genus *Hexagenia* contains the best known of all May flies, whose adults swarm in thousands over lakes and rivers, where, as nymphs, they but recently burrowed in the mud. *Hexagenia bilineata* is probably the most familiar and widely distributed of American May flies. The closely related *Hexagenia recurvata* of this paper is less known, for it emerges from small streams in May or earlier and does not fly in great swarms like the other species. The length of its life is not known; but fairly grown nymphs are common in October and through the winter, though they are rare in June and July. Nymphs of *Hexagenia recurvata* can be recognized by the long mandibular tusks and especially by the unbranched rudimentary first gill (Plate I, Fig. 4); the tusks of *Hexagenia bilineata* are shorter, and the rudimentary first gill is double, like a tuning-fork. Several hundred nymphs used for this and other work were collected from the same region of one stream, mainly from November to February; comparatively few were taken in March and April.

In the laboratory the nymphs were kept in running water in glass finger bowls, bedded with silt and sand from the nymphs' native stream, and tightly covered with cheesecloth. Early in the experiment crowding was found disastrous, and not more than 10 nymphs were usually housed in one bowl. In order to care for a large number of nymphs, five or six bowls were supplied from one faucet, the water being first piped into a large bottle from which it was carried to the individual bowls by glass siphon tubes. At intervals of 2 or 3 weeks scrapings of algae were stirred into the muddy beds of the bowls. The gills were usually removed from nymphs about a week after they were collected. The operated animals and their controls were then kept for varying intervals, sometimes for 5 months or more in the conditions described.

THE GILLS AND GILL MOVEMENTS

The equipment of gills in *Hexagenia recurvata* consists of 7 pairs of tracheal gills, one pair attached to each of the first 7 abdominal segments (Plate I, Fig. 1). On the first segment the gill is rudimentary, straplike, and single and can have little or no respiratory function (Plate I, Fig. 4). Those on segments 2–7 each have two leaflike divisions, the lamellae veined with tracheae and bordered by filaments which are threaded with them (Plate I, Fig. 3). Each pair of gills is jointed to a flexible eminence of the body wall, which bends with it.

Waves of motion pass backward over the gills sometimes too rapidly for the eye to follow but at other times in listless ripples. The gills may cease moving altogether, usually for a few seconds or for 1 or 2 minutes, though at times they may be quiet for half an hour. The motions vary with those of the nymph's body and also with the temperature and chemical content of the water. When resting, the gills are held almost upright or bent slightly backward (Plate I, Fig. 1). When waving, each pair bends downward and backward, then upward again. As a pair bends backward, the filaments of the opposite gills touch each other, instantly separating as the gills are pulled upward, the whole performance resembling the rapid opening and closing of a V. So far as observed, the gills move whenever the nymph is active, especially when it burrows. While digging, the nymph holds its front legs closely against its wedge-shaped head, then lunges forward, at the same time pushing these legs outward. Almost synchronously with this, its middle legs are pushed outward and backward and the hind legs backward. As its body wedges forward, the nymph fans the silt with its gills, opening and closing them in the V-shaped angle. Thus, their surfaces are cleared of the mud thrown back by the hind legs, and the water is continually circulated about them.

COMPARISON OF BEHAVIOR AND GENERAL CONDITIONS OF NYMPHS WITH AND WITHOUT GILLS

Hexagenia nymphs which had their gills removed burrowed little or not at all. They stayed on the surface of the mud, their few spontaneous movements being slow and labored, but not otherwise

PLATE I

FIG. 1.—Dorsal view of full-grown nymph of *Hexagenia recurvata*. Living specimen. Length without setae, 25 mm.

FIG. 2.—Dorsal view of nymph showing black scars where the gills were removed. Gills were removed November 21, 1930; nymph lived till March 4, 1931. Length without setae, 17 mm.

FIG. 3.—Typical gill, with two lamellae, showing tracheae and filaments. Photographed immediately after removal.

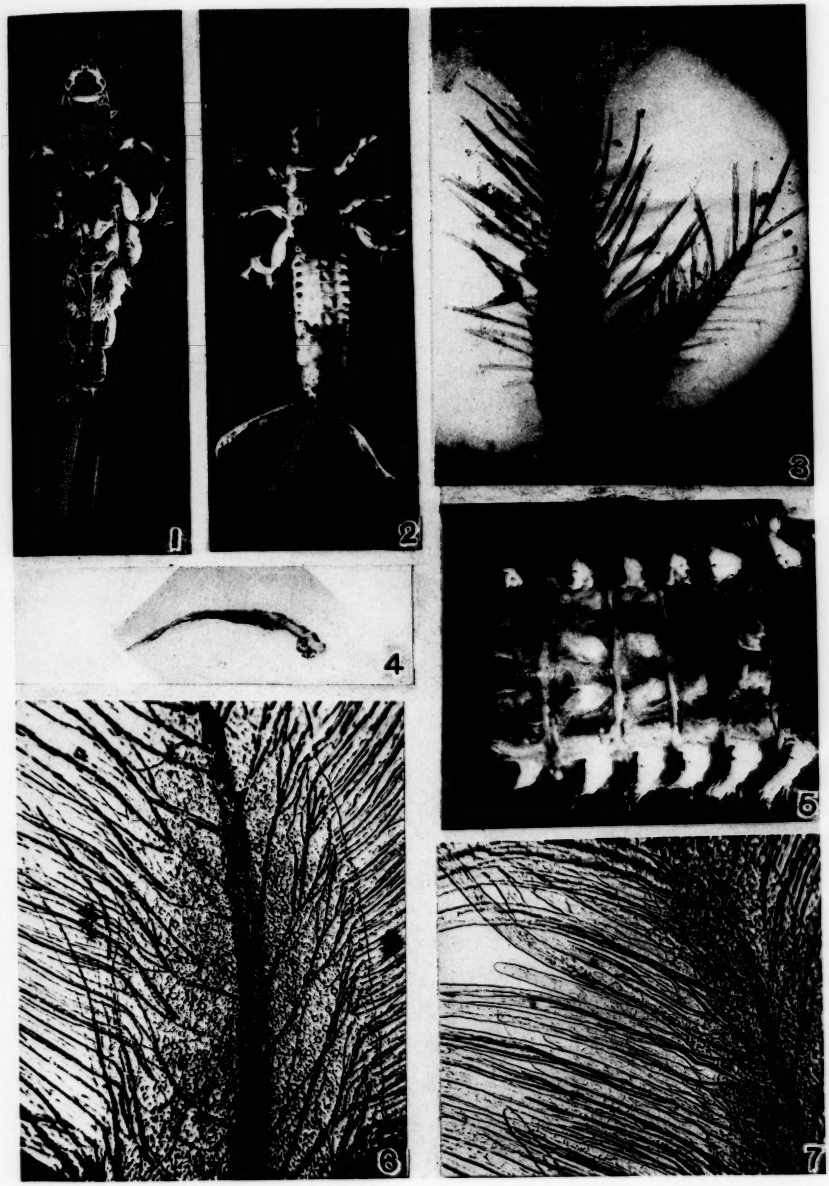
FIG. 4.—Rudimentary first gill.

FIG. 5.—Gill scars of specimen in Figure 2, showing hairs which protect the bases of the gills. The chitin overlapped by the gills is pale or transparent.

FIG. 6.—A section of the gill in Figure 3, showing the tracheal supply near the tip and in the filaments of one side.

FIG. 7.—Another section of the same gill.

PLATE I



strikingly different from those of the normal nymph. Though they were not disposed to burrow, their occasional digging showed that they were not disabled for the performance. In diffused daylight the control nymphs would dig into sand or mud rapidly, usually pausing only after the head and thorax were buried. Taken out of the light, they at once became more leisurely in their burrowing. Gill-less nymphs, on the other hand, showed little or no reaction to daylight, rested, or moved slowly over the mud surface, showing scant favor for the shaded side of the bowl where they were kept.

While gill-less nymphs showed little disposition to swim, they would do so when stimulated. When they took off from the mud surface, their postures were like those of the normal nymphs. So was their undulating progress through the water afterward. Although the difference in speed between normal and gill-less nymphs was striking, motion pictures of the two kinds of nymphs revealed little or no difference in swimming postures or in the preliminary digging of the burrow. When alighting on the mud, however, the gill-less nymphs were apt to land on one side or the other, often to roll over on their backs, showing their failure to balance themselves.

Normal nymphs lived well in the laboratory conditions earlier mentioned; and though a serious mortality often followed operations, nymphs whose gills were removed in November lived 21 weeks, while others operated upon in November and December lived 2 and 3 months. High mortality among the gill-less nymphs began in late winter and rose through February till nymphs operated in early April lived only a few hours. Even normal nymphs which had lived 3 to 4 months in the laboratory could not survive gill-removal in the spring. The greater sensitiveness was undoubtedly correlated with growth and changes preparatory to adult maturity. Normal nymphs were not easily affected by crowding, while the gill-less ones were very sensitive to it. Although no special account was taken of molting, a search for molted skins was made whenever food was put into the bowls. From early November through February, 5 skins were found among about 500 controls, but none were found in the same number of gill-less nymphs examined.

Nymphs that were collected after the middle of February had more prominent wing pads and larger reproductive organs. Such

symptoms of rising metabolism appeared not only in recently collected nymphs but to some extent in those which had been kept in the laboratory. A high mortality after operations also began to occur. Of 110 nymphs which were gill-less on February 23, only 13 were alive 9 days later, and immature nymphs were the only ones which survived at all. Freshly collected nymphs and those of long laboratory residence responded alike to the operation. On the other hand, the nymphs that had had their gills removed earlier in the winter lived on successfully and were mostly the subjects of the spring oxygen and carbon-dioxide tests (Tables I and II).

The control nymphs ate a moderate amount of food while in captivity. The stomachs of those killed from time to time through the year displayed the typical *Hexagenia* diet of diatoms, other algae, and organic débris. Microscopic sections of control nymphs which had been kept in the laboratory 5 months showed stomachs or intestines, or both, fairly filled with food débris. Examinations of gill-less nymphs usually disclosed small amounts of food, and microscopic sections of them showed the food canal only partly filled.

EXPERIMENTS TO DETERMINE THE DIFFERENCE IN OXYGEN INTAKE BETWEEN NORMAL GILLED NYMPHS AND OPERATED GILL-LESS ONES

These experiments were made to determine the importance of the gills in oxygen intake. In order to do this, the oxygen intake occurring when the gills were present was measured and compared with the amount consumed when the gills had been removed for a considerable time.

The oxygen determinations were made according to the Winkler method (Kemmerer *et al.*, 1923, p. 58; Shelford, 1929, p. 495; Theriault, 1925), and the measurements taken in the Thompson-Miller apparatus for the micro-determination of dissolved oxygen (Thompson and Miller, 1928; also Shelford, 1929, p. 495). The procedure with the nymphs was much like that which has been used in similar experiments upon caddis-fly larvae (Morgan and O'Neil, 1931). After its oxygen content had been measured, 200 cc. of water were poured into each of three bottles. A group of 5 normal nymphs was weighed and put into one bottle. Another group as nearly equal

TABLE I

OXYGEN CONSUMPTION OF *Hexagenia recurvata*, IN GROUPS OF 5 GILL-LESS AND 5 CONTROL NYMPHS FOR TWO SUCCESSIVE 5-HOUR PERIODS

Experiment	Date of Collection	No. of Weeks between Collection and Operation	No. of Weeks between Operation and Experiment	Temperature of Water Bath Surrounding Bottles	Weight of Groups of Nymphs in Grams	Cubic Centimeters per Liter of Oxygen in Bottles of Water at Beginning of Experiment, Blank, Control, Gill-less	Cubic Centimeters per Liter of Oxygen in Water after 5 Hours	Cubic Centimeters per Liter of Oxygen in Water after 10 Hours	Actual Oxygen (Cubic Centimeters) Consumption in 10 Hours	Oxygen (Cubic Centimeters) Consumption Calculated for Controls of Same Weight as Gill-less
I.....	11/14/30	1	14	6° C.		b. 9.07	9.07	9.07	.....	.....
					control 0.452	c. 9.07	6.04	5.90	3.17	3.17
					gill-less 0.450	g. 9.07	*	7.34	1.73	1.73
II....	11/14/30	1	16	6° C.		b. 7.33	7.33	7.33	.....	.....
					control 0.324	c. 7.33	6.90	6.20	1.13	0.91
					gill-less 1.260	g. 7.33	6.76	6.76	0.57	0.57
III....	11/14/30	1	16	6.5° C.		b. 8.74	8.74	8.74	.....	.....
					control 0.422	c. 8.74	6.48	5.64	3.10	2.94
					gill-less 0.400	g. 8.74	7.45	7.19	1.55	1.55
IV....	12/1/30	8	5	7° C.		b. 8.59	8.59	8.59	.....	.....
					control 0.408	c. 8.59	6.20	5.35	3.24	2.38
					gill-less 0.300	g. 8.59	7.61	7.33	1.26	1.26
V.....	11/21/30	9	15	6° C.		b. 8.45	8.45	8.45	.....	.....
					control 0.380	c. 8.45	7.75	6.76	1.69	1.25
					gill-less 0.280	g. 8.45	8.03	7.61	0.84	0.84
VI....	11/21/30	9	15	7.5° C.		b. 8.59	8.59	8.59	.....	.....
					control 0.390	c. 8.59	7.19	5.92	2.67	1.85
					gill-less 0.270	g. 8.59	8.17	7.61	0.98	0.98
VII...	12/12/30	10	2	6.5° C.		b. 8.45	8.45	8.45	.....	.....
					control 0.319	c. 8.45	6.76	5.07	3.38	2.75
					gill-less 0.260	g. 8.45	7.33	6.62	1.83	1.83

Total actual oxygen consumption (seven experiments):

	Total	Average
Control nymphs	18.38 cc.	2.63 cc.
Gill-less nymphs	8.76 cc.	1.25 cc.

Total oxygen consumption calculated for controls of the same weight as gill-less nymphs:

Control nymphs	15.25 cc.	2.18 cc.
Gill-less nymphs	8.76 cc.	1.25 cc.

* Five-hour consumption of gill-less nymphs not taken in Experiment I.

as possible in weight, consisting of 5 gill-less nymphs, was placed in a second bottle. The third bottle of water was carried as a control. A hole was made in each of the corks, and a glass tube forced through until it projected well into the water. A piece of rubber tubing was fitted to the other end and clamped tight by a pinchcock. At the beginning of the experiment, after the nymphs were in the water, a piece of wire netting was fitted rather closely down over them. Finally, a layer of paraffin oil was poured on the surface of the water

TABLE II

COMPARISON OF ACTUAL OXYGEN CONSUMPTION OF CONTROL GILLED AND GILL-LESS NYMPHS DURING THE FIRST AND THE SECOND 5 HOURS OF OXYGEN CONSUMPTION (SEE TABLE I)

EXPERIMENT	CONTROL NYMPHS			GILL-LESS NYMPHS		
	First 5 Hours	Second 5 Hours	Total	First 5 Hours	Second 5 Hours	Total
I	3.03 cc.	0.14 cc.	3.17 cc.	.....	.....	1.73* cc.
II	0.43 cc.	0.70 cc.	1.13 cc.	0.57 cc.	0.0 cc.	0.57 cc.
III	2.26 cc.	0.84 cc.	3.10 cc.	1.29 cc.	0.26 cc.	1.55 cc.
IV	2.39 cc.	0.85 cc.	3.24 cc.	0.98 cc.	0.28 cc.	1.26 cc.
V	0.70 cc.	0.99 cc.	1.69 cc.	0.42 cc.	0.42 cc.	0.84 cc.
VI	1.40 cc.	1.27 cc.	2.67 cc.	0.42 cc.	0.56 cc.	0.98 cc.
VII	1.69 cc.	1.69 cc.	3.38 cc.	1.12 cc.	0.71 cc.	1.83 cc.
Total (Exps. II–VII)	8.87 cc.	6.34 cc.	15.21 cc.	4.80 cc.	2.23 cc.	7.03 cc.
Average	1.48 cc.	1.06 cc.	2.54 cc.	0.80 cc.	0.37 cc.	1.17 cc.

* Five-hour consumption of gill-less nymphs not taken in Experiment I.

in each bottle, to keep oxygen from entering or escaping. The corks and tubing connections were then sealed over with paraffin. All the bottles were kept in a water bath during the experiment (Table I, temperature of water bath).

Samples of water for determination were taken at the beginning and after the fifth and tenth hours of the experiment. Air was prevented from entering by technique of manipulation possible after practice with the Thompson-Miller apparatus.

The amounts of oxygen consumed by nymphs in seven experiments, each dealing with 5 control gilled and 5 gill-less nymphs, are given in Table I.

Most of the gill-less nymphs used in the experiments (Table I)

had had their gills removed 14 or 16 weeks before; 10 nymphs had their gills removed 2 and 5 weeks before. No regeneration occurred. *Hexagenia* nymphs are naturally vigorous, sometimes jerky, in their motions including those of the gills. Since all movements are obviously correlated with the amount of oxygen consumed, it was necessary to take care to have the nymphs as quiet as possible during the experiments.

In every experiment the oxygen consumption of the normal nymphs for the 10 hours greatly exceeded that of the gill-less ones (Table I). In the first 5 hours the total oxygen consumed by the gilled nymphs was almost twice that of the gill-less ones; in the second 5 hours it was nearly three times as great. The oxygen consumption of the gill-less nymphs was only one-half as much in the second as in the first 5 hours (0.8 cc. in first 5 hours to 0.4 in second 5 hours). The oxygen consumption of the gilled nymphs was slightly less in the second than in the first 5 hours (1.5 cc. in first 5 hours to 1.1 in second 5 hours) (Table II).

In considering the unevennesses of oxygen consumption in individual experiments, it should be remembered that some movement of the nymphs was unavoidable. The length of time between gill-removal and experiment seemed to make no difference in the amount of oxygen that the nymphs consumed. Compare experiments III and IV, VI and VII (Table I). A few nymphs which were tested in the spring, a day or two after gill removal, consumed the same amount of oxygen as those that had lived several weeks after the operation. The series of tests showed that, although *Hexagenia* nymphs can live through the winter season without gills, their oxygen consumption is cut down about one-half.

INDICATOR TESTS TO COMPARE THE RATE OF CARBON-DIOXIDE ELIMINATION IN NORMAL AND GILL-LESS NYMPHS

These tests were made to find out whether the gills were relatively as important in carbon-dioxide elimination as in oxygen intake. They were performed by Fox's (1921) indicator method.[1] Three

[1] Enough potassium hydroxide was added to a weak solution of aqueous alkaline haematoxylin to change the color of the solution from pink to bluish pink. As carbon dioxide is mixed with this non-toxic solution, its color changes from bluish pink to orange and finally to a pale yellow.

vials, 40 mm. long and 25 mm. in diameter, were filled with very dilute alkaline haematoxylin solution. A normal nymph was placed

TABLE III

RESULTS OF INDICATOR TESTS SHOWING THE COMPARATIVE RATE OF CARBON-DIOXIDE ELIMINATION IN NORMAL AND GILL-LESS NYMPHS*

In each experiment one vial contained a control nymph and one a gill-less nymph. For the third vial, carried as a control, no record is shown here.

Exp.	Date of Collection	No. of Weeks between Collection and Operation	No. of Weeks between Operation and Experiment	Minutes Required To Bleach Indicator Solution		Ratio of Time of Gill-less to Control
				Control	Gill-less	
I	11/10/30	1	23	6	15	2.5
II	11/14/30	1	23	8	16	2.0
IV	11/14/30	1	23	5	18	3.6
V	11/14/30	1	23	6	15	2.5
VI	11/14/30	1	23	7	17	2.4
Total for experiments				32	81	
Average				6.4	16.2	

SAME TESTS MADE UPON NYMPHS COLLECTED IN SPRING AND RECENTLY DEPRIVED OF GILLS

Exp.	Date of Collection	No. of Days between Collection and Operation	No. of Days between Operation and Experiment	Minutes Required To Bleach Indicator Solution		Ratio of Time of Gill-less to Control
				Control	Gill-less	
VIII	4/18/31	12	1	4.0	17.0	4.3
IX	4/18/31	12	1	3.5	17.5	5.0
X	4/18/31	12	1	4.5	16.0	3.5
XI	4/18/31	12	1	4.0	16.0	4.0
XII	4/18/31	12	1	3.0	15.0	5.0
Total for experiments				19.0	81.5	
Average				3.8	16.3	

* Two experiments made upon nymphs with the same history as in Experiment II but immersed in more dilute indicator solutions required, respectively, 13 and 10 minutes for the normals and 28 and 28 minutes for the gill-less nymphs to "bleach" them. The ratio of time taken, gill-less to control, was 2.2 and 2.8.

in one vial, a gill-less one of the same weight in another, and the third was carried as a blank control. The corks were sealed with

paraffin, and observations were made immediately. The vials were placed against white paper, and observations continued until the solutions were "bleached" to pale yellow. In the first set of tests made upon nymphs that had lived for 6 months in the laboratory, the normal animal bleached the solution two to three times faster than the gill-less ones. In the spring the same tests were repeated upon nymphs whose gills had been cut off the day before (Table III, Exps. VIII–XII). Although gilled and gill-less nymphs of the same weight were selected as before, the gilled nymphs bleached the solution about four times faster than the operated ones. In neither series of tests was there any indication of "bleaching" localized about the spiracles.

DISCUSSION

The observations on the gill-less *Hexagenia* nymphs indicated that their gills were, first of all, important respiratory organs whose absence was responsible for the marked decrease in oxygen consumption and carbon-dioxide elimination shown in the tests. This was further apparent in their consistently slow motions and general inactivity.

Some of their motions, especially swimming, were hampered by the lack of mechanical aid from the gills; but even these were usually executed in good form. But their sluggishness and inertia were evidently expressive of the changed metabolism resulting from the decreased exchange of gases. It seemed altogether likely that the gill-less nymphs' positive reaction to light was correlated with the excess carbon dioxide accumulated within them (Allee and Stein, 1918). It is true that these nymphs ate less than normals and that a hungry condition has more than once been the apparent cause of reversed phototropism in other animals. Stier (1926) found this in the newt *Triturus viridescens*. But it is also true that, almost immediately after their gills had been removed, these nymphs changed from photo-negative to photo-positive even when they had but recently taken food and still had it in their stomachs. In experiments which Wodsedalek (1911) performed on the May fly *Heptagenia interpunctata*, he found that the normal nymphs were practically all negatively phototropic. But as soon as carbon dioxide was bubbled through the water, a large percentage of them became positively phototropic.

Natural reversal of phototropism preceding emergence is common in May flies and in other aquatic insects. Nymphs of the May flies *Epeorus* and *Iron* live on the under sides of rocks, ordinarily never coming to the lighted side. But, just before their emergence, they move onto the lighted side by hundreds. Their nymphal covers are thick then, the tracheae of the gills are beginning to be cut off from those of the body, and the gill is losing its function. It seems probable that their condition is similar to that of the gill-less *Hexagenia* nymphs, and their reaction the same.

Various suggestions have been made concerning the passageways of gases in nymphs that are living without their gills. Cuénot (1925) believed it possible that his nymphs of *Cloëon dipterum* went to the surface of the water and took air through their thoracic spiracles. Remy (1925) thought that May fly nymphs, when deprived of their gills, depended for some time on the supply of air that had been in their tracheae before the operation. He cited examples of aërial insects which were able to survive 4 to 5 days' immersion in water by so doing. He further explained that, in summer, May flies survived gill amputation but a short time because they exhausted their tracheal air supply so much more quickly than in winter. He felt it unnecessary to ascribe tegumentary or intestinal respiration to them.

The gill-less *Hexagenia recurvata* nymphs were never observed coming to the surface for air or in any other way relying upon their spiracles. As already mentioned, during the carbon-dioxide experiments the solution about the spiracle region was bleached no more rapidly than elsewhere. Sections of *Hexagenia* nymphs that have thus far been made indicate that the spiracles are closed. However, this preliminary impression should in no way be regarded as a conclusion. The open or closed nature of the spiracles can only be decided by thorough morphological work. Nevertheless, in the present studies the behavior of the gill-less nymphs, as well as the results of the carbon-dioxide experiments, indicated that exchange of gases took place through the body wall.

No regeneration was observed among the approximately 500 gill-less nymphs under observation at some time during the experiments (Plate I, Figs. 2, 5). Cuénot (1925) found that nymphs of *Cloëon dipterum* regenerated their gills freely, and several other investiga-

tors have reported the same thing in various species of May flies. In collected material it is not uncommon to find regenerating gills on nymphs of various species. Such specimens of *Hexagenia recurvata* were collected during the present study. The failure of *Hexagenia* to regenerate in the laboratory could not have been associated with the age of the nymphs, for several sizes were used and many were but half-grown when their gills were removed. (Full-size nymphs are 24–28 mm. long, without caudal setae.) This may have been due to the thoroughness with which the base of the gill was removed.

Hexagenia nymphs lived for a long time without their gills if they were removed in the early winter season of lowered activity. Nymphs whose gills were removed in November lived about 5 months; those that underwent the same operation in early spring lived but a week or two, and in late spring only 2 or 3 days or even less. Those that lost their gills in November lived through the season of low activity very easily, but in the spring they suddenly began to die in large numbers. The fate of these nymphs and the swift mortality that followed the spring operations showed that gills are essential to the full activity of life in these May flies. Their dependence on their gills is in striking contrast to the caddis fly, *Macronema zebratum*, whose larvae not only lived months after their gills were removed but pupated and emerged more or less successfully (Morgan and O'Neil, 1931). This difference in the importance of the gills of *Hexagenia* and *Macronema* is also suggested by the ratios of oxygen intake of gilled and gill-less animals in the two forms. Results of a series of tests on *Macronema* showed no significant difference between the average total oxygen consumption of normal and gill-less larvae (Morgan and O'Neil, 1931). On the other hand, control *Hexagenia* nymphs consumed twice as much oxygen as gill-less ones (Table I).

SUMMARY

1. In *Hexagenia recurvata* the tracheal gills are respiratory organs that are essential to life. Nymphs that lived without gills through the winter period of lowered activity were unable to survive through the spring. Nymphs whose gills were removed in spring died very soon, often within a few hours.

2. The oxygen intake of normal nymphs was approximately twice as much as that of gill-less ones.

3. Normal nymphs eliminated carbon dioxide more than twice as fast as gill-less ones.

4. In gill-less nymphs, exchange of gases appeared to occur through the body wall. There was no physiological evidence of open spiracles.

5. Normal nymphs are generally photo-negative except before emergence. After gill-removal they became photo-positive, the condition appearing to correlate with their content of carbon dioxide.

6. Nymphs that lived through the winter without gills were inactive, made few spontaneous movements, and rested upon the surface of the mud, whereas their controls were active and burrowed freely. The swimming and burrowing movements of gill-less nymphs were slow but not different in form from those of normal nymphs.

7. From November to March control nymphs molted very rarely; gill-less ones, not at all.

8. No regeneration of the gills was observed on the experimental animals, though a few specimens with regenerating gills were found in collected material.

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THE VISCOSITY OF THE PLASMAGEL OF AMOEBA PROTEUS AT DIFFERENT TEMPERATURES

(One figure)

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COLLOIDAL materials show a wide range of variation in their relation to temperature. In some colloidal solutions an increase in temperature is accompanied by a sharp decrease in viscosity as the solution changes from the state of a gel of high viscosity to the state of a sol of low viscosity. However, the viscosity of many colloidal solutions diminishes more or less gradually with a rise of temperature; such solutions are relatively fluid at all ordinary temperatures. The colloids of a living system may show a behavior which is unlike that of inanimate materials in general. Thus Heilbronn (1922) found that the protoplasm of the slime mold *Reticularia* passed through a maximum viscosity as the temperature was raised. Similar results were obtained by Heilbrunn for the protoplasm of *Cumingia* eggs (1924) and also for *Amoeba dubia* (1929). In other cases the behavior of the protoplasmic colloid corresponds more nearly to that of inanimate materials, in that a progressive decrease in viscosity has resulted from a rise in temperature. This type of behavior has been reported by Fauré-Fremiet, F. and G. Weber, and Pantin. References to these authors, as well as a more complete review and discussion of the literature, may be had by referring to a monograph by Heilbrunn (1928).

In the present experiment the method employed for the determination of viscosity was the centrifuge method as used by Heilbrunn (for references see Heilbrunn, 1928). The centrifuging was done on the high speed of an old model Bausch and Lomb hand centrifuge, which was turned at the rate of one turn per second. At this speed there was developed a centrifugal force 4,084 times gravity. In order to arrive at a relative measurement of the viscosity, a scale of arbitrary units was selected in the following manner. When amoebae

are centrifuged a few seconds (10 seconds or less) at the foregoing speed, the crystals of the inner protoplasm, or plasmasol, become massed at one end of the amoeba. On the other hand, the crystals of the outer protoplasm, or plasmagel, are still distributed throughout this region. If the time of centrifuging is increased by several seconds, the crystals in the outer protoplasm will likewise be displaced toward one end of the amoeba. Eventually, when the time has been increased sufficiently, the crystals of the plasmagel will have been displaced to such an extent that one-half of the amoeba no longer contains any crystals within its protoplasm. On the basis of this criterion, the viscosity can be designated as the centrifuging time, in seconds, which is necessary in order to displace the crystals of the plasmagel to such an extent that one-half of the plasmagel no longer contains any crystals in its protoplasm. In the determination of the normal viscosity at room temperature (22.9°) it was found that in 75 seconds the granules were not displaced halfway but that in 80 seconds half of the protoplasm contained no crystals at all. Hence, a value of 80 was assumed to be the normal viscosity at that temperature; and in order to make the results consistent, a culture which did not subsequently give a preliminary value of 80 at 22.9° was not used. Within a given culture at any one time, the behavior of the amoebae is quite constant; but it is to be noted that different cultures may vary rather widely in their viscosity or that the viscosity of the same culture may vary on different days. It is thus quite essential that a preliminary test should be taken before each experiment. Less variation is encountered if all of the specimens are obtained from one culture. Two cultures were used during the course of this experiment. In making tests, one should reach an early decision upon the proper end-point and maintain it throughout the course of the experiment. For example, in this experiment a given test was not considered as representing the viscosity value unless all the crystals were displaced halfway. There is, however, one exception to this rule, in that occasionally crystals become lodged in small projecting pseudopodia and cannot be displaced by any amount of centrifuging. One can soon learn to recognize such specimens by focusing carefully at the tips of the projecting pseudopodia. It is very often found that when one examines the slide the amoebae

appear not to have been centrifuged at all. Such specimens are being observed from the polar view and need only to be turned so as to be observed from the side. This can be accomplished with a little practice either by tilting the microscope or by flipping the slide with the finger nail. Table I is a representative data sheet which illustrates the manner of arriving at a given value.

TABLE I

TESTS OF VISCOSITY AT 16.2°–16.3°

Culture 1, April 6, 1931; viscosity, 100 at 16.3°

Immersion Time in Seconds	Number Observed	Displacement of Crystals	Centrifuging Time in Seconds
60	3	None halfway	90
60	5	One halfway, 4 not	90
60	.....	Uncertain	90
60	1	None halfway	90
60	3	None halfway	90
60	4	Three halfway, 1 moving	95
60	3	One halfway, 2 not	95
60	2	None halfway	95
60	1	None halfway	95
60	2	None halfway	95
80	2	None halfway	95
60	5	Three halfway, 2 moving	100
60	.....	None found	100
60	3	Two halfway, 1 not	100
60	5	Three halfway, 1 not, 1 moving	100
60	2	Two halfway	100
60	4	Four halfway	100
62	.....	Uncertain	100
60	2	Two halfway	100
107	4	Four halfway	100

It should be clearly understood that there are certain difficulties in the use of the centrifuge method for the study of the viscosity of the plasmagel of *Amoeba proteus*. A possible error lies in the fact that some of the crystals of the plasmagel may lie very close to the outer limiting membrane of the cell. The dislodgment of the crystals from such a position may depend on other forces than the viscosity of the plasmagel itself. Another, and perhaps a more serious error lies in the possibility that the thickness of the plasmagel may vary with changes in external conditions. In all the measurements recorded in this paper it is assumed that the thickness of the plasmagel

does not change markedly with change in temperature. At present there is no certain method of measuring the thickness of the plasmagel. If such methods are developed, it may later be possible to revise the data as here presented. The facts themselves are certain enough. With change in temperature, there is a pronounced variation in what Heilbrunn and Daugherty (1932) refer to as the "centrifugal value," that is to say, the number of seconds required to shift the crystals of the plasmagel.

TABLE II

VISCOSITY OF THE PLASMAGEL OF *Amoeba proteus* AT DIFFERENT TEMPERATURES

Temperature (Degrees Centigrade)	Minimum Time of Immersion (Minutes)	Viscosity or Centrifuging Time in Seconds	Number Observed	Date
0.4	60	115	95	4/ 9/31
2.1	55	120	61	4/ 2/31
4.5	60	125	52	4/10/31
8.9	60	120	73	4/ 7/31
11.2	60	110	72	4/ 6/31
16.3	60	100	39	4/ 6/31
19.8	60	90	30	3/31/31
22.9	60	80	23	3/31/31
25.4	60	75	83	4/ 8/31
29.9	34	60	22	3/ 8/31
32.3	56	50	18	3/13/31
33.7	92	35	14	3/14/31
35.5	60	15	14	3/27/31
38.3	60	.....	All dead	4/ 3/31

The amoebae were brought to the desired temperature by transferring them from the culture to capillary tubes, which were then partially immersed in water contained in larger tubes fitted with corks. The larger tubes were continually surrounded by the water of the thermostat at the desired temperature of the experiment. Sample tests showed that within 20 minutes the water of the capillary tubes could be raised from 2° to 35° by immersing them in the bath. In most cases, however, they were immersed for a period of 1 hour. By placing the tubes in the bath at intervals of 10 minutes, tests can be made on a series of amoebae all of which were immersed for the same length of time. At the end of the period of immersion the capillary tubes were removed, dried by wiping with a cloth, and centrifuged without further delay. This whole procedure must be

done very rapidly in order to reduce the element of error which exists in the difference between room temperature and the temperature of the bath, although no difficulties of this sort were encountered in this experiment. The results are summarized in Table II, in which each row represents a series of values such as that illustrated in Table I.

Inspection of the viscosity-temperature curve shown in Figure 1 shows that it is composed of three distinct divisions. The major portion of the curve is in the form of a straight line which indicates that between 5° C and 30° C the viscosity of the plasmagel decreases progressively with a rise in temperature. It is evident that between these temperatures the outer protoplasm of *Amoeba proteus* is a comparatively viscous material which is consistently becoming more and more fluid as the temperature increases. Evidence for this same type of curve was also obtained by Pantin (1924) for the immature eggs of *Nereis*. Heilbrunn (1921) has pointed out that the unfertilized eggs of *Nereis* are also relatively viscous at ordinary temperatures. On the other hand, it has been shown that some types of protoplasm pass through a maximum viscosity as the temperature is raised. This has been demonstrated by Heilbronn (1922), working with slime molds, and also by Heilbrunn (1924 and 1929), with *Cumingia* eggs and *Amoeba dubia*. All of these are relatively fluid at ordinary temperatures. From these observations it would seemingly appear that the essential difference between those types of protoplasm which show a progressive decrease with changes in temperature and those which pass through a maximum viscosity is a difference in the relative viscosity at ordinary temperatures.

However, it should be pointed out that Pantin made observations at intervals of 10° and thus has only four points on his curve. It is interesting to note that between 10° and 20° the percentage drop of the protoplasmic viscosity is of nearly the same magnitude as the percentage drop for the viscosity of water, but that at higher temperatures the protoplasmic viscosity undergoes a sharp decrease which is quite unlike the behavior of water. This sudden change is most evident on the curve between 30° and 35°. At 38.3° the amoebae die when immersed for a period of 1 hour. No measurements of viscosity could be obtained at this point, but it is to be assumed that

the viscosity was very low, since the granules had settled out under the influence of gravity. The maximum viscosity is shown at 4.5°; further decrease in temperature resulted in a slight drop in viscosity

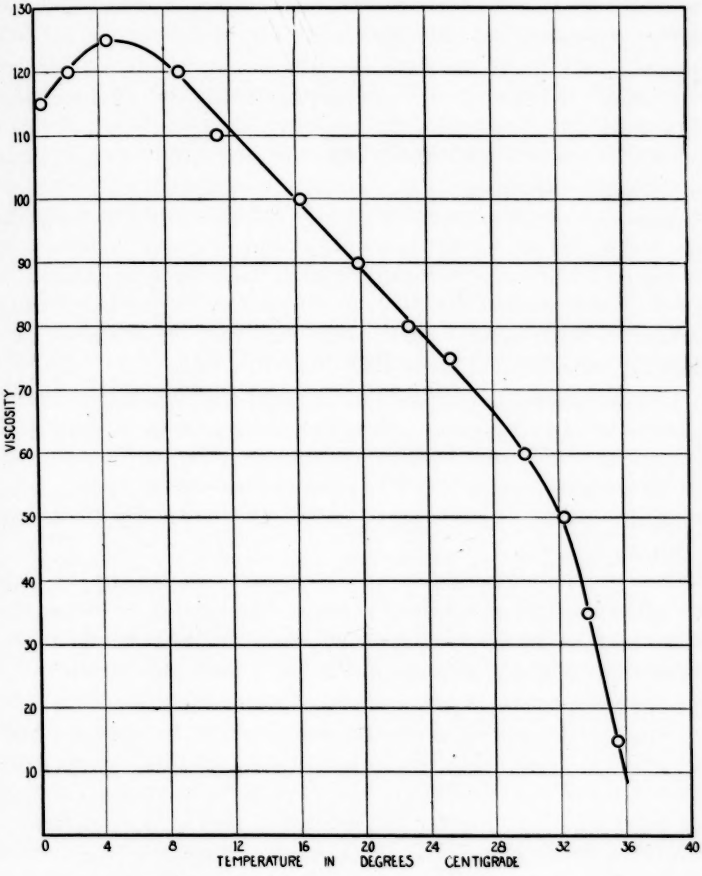

FIG. 1.—Viscosity-temperature curve of plasmagel of *Amoeba proteus*

for the only two readings which were obtained. One of these readings (2.1°) was obtained by taking the amoebae from the water bath at a temperature of 2.1° and centrifuging them at room temperature,

which was much higher. However, in view of the fact that the reading is consistent with a later reading at 0.4°, which was obtained in a cold room (about 8°), the result is probably valid and has been included on the curve. The significance of this drop is not yet apparent; it may be that some correlation can be drawn with the fact that water has its greatest density at 4° C.

Inasmuch as the speed of displacement of the granules is in part a function of the difference between the specific gravity of the granules and that of the medium surrounding them, it might be thought that a change in the specific gravity of the water, by causing a corresponding change in the specific gravity of the fluid surrounding the granules, would in this manner account for the observed differences in the speed of displacement of the granules. This, however, is unlikely in view of the fact that the differences in the specific gravity of water at different temperatures are so slight that they could scarcely account for the relatively great differences observed.

It has been stated elsewhere in this paper that living protoplasm is probably of two general types. Protoplasm which is viscous at ordinary temperatures probably responds to increases in temperature by showing a progressive decrease in viscosity, but protoplasm which is ordinarily fluid behaves differently by passing through a maximum viscosity.

Amoeba proteus is perhaps somewhat peculiar in that the cortex is a gel of relatively high viscosity at ordinary temperatures, while the inner region or plasmasol is more or less fluid, as evinced by the readiness with which it flows. Preliminary tests indicate that the plasmasol is a region of low viscosity, and probably it responds to temperature changes in much the same manner as does the protoplasm of *Amoeba dubia* (Heilbrunn, 1929). The plasmagel, however, is a region of high viscosity and gives the same type of curve as was obtained by Pantin for the immature eggs of *Nereis*. Hence, there occur within one animal, two kinds of protoplasm—a gel and a sol—which differ in respect to their colloidal properties and likewise the manner in which they respond to physical changes in their environment.

Eventually these results may be of interest in interpreting the speed of amoeboid movement at different temperatures.

This work was done under the direction of Dr. L. V. Heilbrunn at the Zoölogical Laboratory of the University of Pennsylvania.

SUMMARY

1. A curve has been plotted in which the relation of temperature to viscosity in the plasmagel of *Amoeba proteus* has been shown as measured by the centrifuge method. The maximum viscosity occurs at 4.5°. Between 4.5° and 30° the viscosity decreases progressively with a rise in temperature. At 30° there is a break in the decrease, and from this temperature to the death-point the viscosity decreases more rapidly.

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THE ACTION OF SODIUM, POTASSIUM, CALCIUM, AND MAGNESIUM IONS ON THE PLASMAGEL OF AMOEBA PROTEUS[1]

(One figure)

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IN A recent paper (Heilbrunn and Daugherty, 1931), it was shown that sodium and potassium ions increased the protoplasmic viscosity of *Amoeba dubia*, and that calcium and magnesium ions had the opposite effect and caused a lowering of the protoplasmic viscosity of this amoeba. These facts are in agreement with older studies of the effects of the common cations of protoplasm on its viscosity, and they find a ready explanation if one assumes that the protoplasmic micellae are charged positively.

Amoeba proteus is a form which closely resembles *Amoeba dubia;* indeed, the morphological differences between the two species were not recognized until 1916. Beyond much question, the essential properties of the protoplasm are the same in the two species. And yet, at first sight, the two amoebae behave very differently toward centrifugal treatment. When *Amoeba dubia* is centrifuged at low speed, with a force 128 times gravity, the crystals of its protoplasm move through the cell in a few seconds, and the amoeba is sharply divided into a clear half and a half filled with crystals. On the other hand, when *Amoeba proteus* is centrifuged at the same speed, a very different appearance is obtained. A hasty observer might conclude that the amoeba had not been affected at all. But after a moment's focusing, it is easy to see that the crystals in the interior of the amoeba are shifted by the centrifugal force. There remains only a narrow ring of crystals which completely envelops the amoeba and tends to obscure the movement of crystals in the interior. As far as one can judge (see Heilbrunn, 1929), the absolute viscosity of the interior protoplasm of *Amoeba proteus* is the same as that of *Amoeba*

[1] This investigation was aided by a grant from the Research Committee of the University of Pennsylvania.

dubia. The difference lies in the fact that there is an outer ring of crystals which does not move when the amoeba is centrifuged slowly. In *Amoeba proteus*, therefore, it is easy to distinguish two regions of the protoplasm: an inner fluid mass which constitutes the main body of the cell, and a thin outer cortex which is relatively solid. This outer cortex may be called the "plasmagel." But it does not correspond exactly to the plasmagel of Mast (1926). This observer defines the plasmagel as a layer of relatively solid substance which surrounds the interior fluid protoplasm of the amoeba and which includes a hyaline plasmagel (commonly called "ectoplasm") and a granular plasmagel. The granular plasmagel is distinguished from the granular plasmasol within it by the fact that there is no streaming movement in the plasmagel. Mast believes that the granular plasmagel may be wide and may include a large fraction of the volume of the amoeba. Our observations indicate that when *Amoeba proteus* is centrifuged at slow speeds, there is only a very narrow ring of crystals that does not move. Thus from experiments with the centrifuge we should be inclined to postulate a much narrower plasmagel than Mast has been led to suppose from his observations on streaming. It should be pointed out that lack of streaming movement does not necessarily imply rigidity. When a liquid flows through a tube, the layer of the liquid in immediate contact with the walls of the tube may not move at all. Hence it is probable that the width of the gel in the cortical region of the amoeba is somewhat less than one might be led to conclude from a study of the protoplasmic movements. If we therefore retain Mast's term "plasmagel," it should be noted that we are referring to a somewhat narrower layer than that which Mast designates as plasmagel. We shall use the term to refer to that portion of the granular plasmagel of Mast in which the crystals do not move when the amoeba is centrifuged at slow speeds.

Although the crystals of the plasmagel are not moved by low centrifugal speeds, they may be shifted if the amoeba is centrifuged rapidly enough. Thus, when the amoeba is centrifuged with a force 4,084 times gravity, after a time the crystals of the plasmagel become displaced in a centrifugal direction until finally half of the cell is free of crystals. The amoeba then presents the appearance shown

in Figure 1. The number of seconds of centrifugal turning necessary to produce this effect is remarkably constant for a given culture of amoeba at any one temperature. Hundreds of tests have shown that at room temperatures with a centrifugal force 4,084 times gravity, the amoebae must be centrifuged for from 80 to 105 seconds to reach the end-point described. Different cultures vary; but in a given culture at a given time, there is remarkably little variation in the individual amoebae. Generally speaking, the variation is not greater

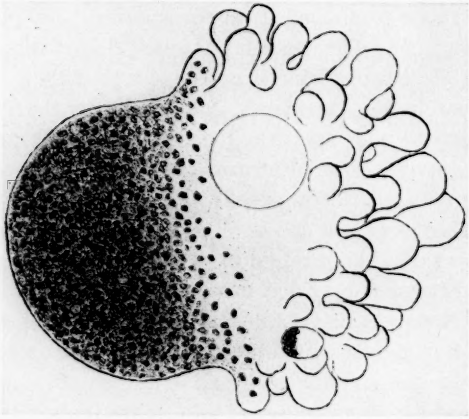

FIG. 1.—A centrifuged amoeba with the crystals in one half of the cell

than 5 seconds, so that if one series of tests shows that 90 seconds is necessary to produce a halfway shift of the crystals, another series of tests will give the same result. However, it should be noted that as a culture ages, there may be a slight increase in the time required to produce the shifting of crystals.

In view of the fact that the number of seconds required to move the crystals is a constant value under a given set of conditions and is a value that may readily be determined, it would seem that centrifugal tests should prove an excellent measure of the viscosity. Certainly the centrifugal tests do give an objective measure of changes in the plasmagel, for, as will be indicated later, the number of seconds required for the crystals to shift varies greatly from one set of condi-

tions to another. And doubtless, too, there is some relation between the speed of movement of the crystals and the fluidity of the plasmagel. But it can scarcely be held that the centrifuge tests provide a quantitative measure of the viscosity of the plasmagel in the same way that centrifuge tests provide a quantitative measure of the viscosity of the main mass of the protoplasm of the cell. When the centrifuge method is used for the determination of protoplasmic viscosity, the speed of particles through the protoplasm is measured. This speed is, in accordance with Stokes' law, an inverse measure of the viscosity (for details, see Heilbrunn, 1928, chap. iv). But in the case of the plasmagel of the amoeba, we are faced with a somewhat different set of conditions. The plasmagel is a shell surrounding the fluid interior. When the crystals in the plasmagel are subjected to centrifugal force, they may move along the sides of the shell or they may be moved out of the shell into the more fluid protoplasm of the interior. Once in the plasmasol, their movement would be much more rapid. Now it is probable that many of the crystals of the plasmagel actually do move into the plasmasol when the cell is centrifuged, for in a series of tests in which amoebae are centrifuged for varying lengths of time, with increase of the time of centrifugal treatment there is not a progressive increase in the width of a clear crystal-free area which gradually extends to half the diameter of the cell. Rather, as the centrifugal treatment is made longer, there is a gradual diminution of the crystals scattered through the lighter half of the cell. Apparently these gradually become detached from the plasmagel and are then shifted.

It seems reasonable to suppose that, when the plasmagel is stiffer, there is more resistance to the shifting of crystals under the influence of centrifugal force. If it takes a longer time for the crystals to be moved, we may be reasonably certain that the plasmagel is stiffer. But it is quite possible, or even probable, that the plasmagel may change its volume at the expense of the plasmasol. If the plasmagel were to increase its thickness, this might tend to retard the shift of the crystals toward one end of the cell. All we can say is that when it takes longer for the shift of crystals to occur, this is an indication that there is an increased gelation in the plasmagel. This increased gelation may be due to a stiffening of the plasmagel or to an increase

in the volume of the plasmagel at the expense of the plasmasol. Perhaps both changes go hand in hand, for any factor which tends to increase the rigidity of the plasmagel might also favor the production of plasmagel from plasmasol.

In whatever way we choose to interpret the results of the centrifuge tests, the facts themselves are certain enough and are easily confirmed from one day to the next. Numerous factors influence the readiness with which the crystals contained in the plasmagel are shifted toward the centrifugal pole. Beyond any doubt, the cortical protoplasm of the amoeba undergoes marked changes in physical state, and these changes are reflected in the ease with which the crystals of the plasmagel are shifted to one side of the cell. If a relatively shorter exposure to a given centrifugal force is sufficient to produce a shift of crystals, then we may well assume that the cortical protoplasm of the amoeba has altered its state in the direction of greater fluidity. In the following account of our experiments, we shall frequently refer to "centrifugal values" for the protoplasm of the plasmagel, and we shall define the "centrifugal value" as the number of seconds exposure to a centrifugal force 4,084 times gravity which is necessary to shift all the crystals of the amoeba into half of its volume. No doubt the centrifugal value is in the main a measure of the viscosity or plasticity of the plasmagel. But in our experiments it can scarcely be regarded as an accurate quantitative measure. Nor can it be supposed that if the centrifugal value is doubled, this indicates an exact doubling of the viscosity of the plasmagel.

In the present study, we propose to discuss the effect of the common cations of protoplasm on the centrifuge value of the plasmagel. The two ions which have the most pronounced effect are potassium and calcium. Potassium produces a very marked lowering of the centrifugal value; calcium, an increase. Magnesium also lowers the centrifugal value. Sodium has but little effect but does consistently produce a slight lowering.

The amoebae used in the experiments were raised from strains obtained originally from Dr. J. A. Dawson, of the College of the City of New York. Many subcultures were made, but, owing to the large number of amoebae used in the tests, it was frequently necessary to purchase new stocks from Dr. Dawson. It is a simple matter to

make centrifuge tests of the plasmagel of *Amoeba proteus*. The ordinary technique of the centrifuge method is used, and the amoebae are centrifuged in glass tubes approximately 4 mm. in outer diameter. When the amoebae are taken from the cultures and placed in these tubes, they round up. There is perhaps an error involved in this change in shape of the amoebae, but the error is a more or less constant one. Moreover, in making comparison centrifuge tests, it is of considerable advantage to have the amoebae in roughly spherical form. The centrifuge used in the experiments was an old model Bausch and Lomb instrument. With the high-speed handle, it developed a force 4,084 times gravity when the handle was turned at the rate of one turn per second.

As stated previously, the centrifuge value is the number of seconds required to give an end-point like that shown in Figure 1. This figure shows an amoeba in which all the crystals have been shifted into one half of the cell, with the exception of a few imprisoned in a pseudopod. In actual practice, if only two or three crystals remained in the clear half of the cell, this was not regarded as significant; but if as many as five or six crystals remained, this was taken to mean that the centrifugal value had not yet been reached. Usually, all the amoebae centrifuged for a given length of time showed approximately the same degree of shift of crystals; but as the end-point was approached, variation might appear. Thus, for a given test, one amoeba might show a halfway shift of crystals, while another might still have a few crystals left in the lighter part of the cell. In such instances, counts were made of the number of amoebae which showed the end-point and the number which did not, and the condition of the majority was taken as indicating whether or not the centrifugal value had been reached.

In testing the effects of the common cations, solutions of the chlorides were used. The salts were dissolved in water redistilled from a fused silica still (Kendall conductivity still). Salts with monovalent cation were made up in m/40 solution; those with divalent cation, in m/60 solution. Osmotically, these solutions are roughly equal, inasmuch as the salts with divalent cations dissociate into three ions instead of two. Actually, the m/40 solutions of the salts with monovalent cations are a trifle stronger osmotically, owing to the greater

activity of the ions. But the difference is not great; and, as a matter of fact, the osmotic effect of such dilute solutions may very well be neglected. Indeed, tests showed that essentially the same results were obtained when the osmotic concentration of the solutions was varied rather widely, as long as the solutions remained sufficiently dilute. The pH of the solutions was always between 6.7 and 7.0, and no buffers had to be used.

The amoebae to be studied were introduced directly into solutions of the salts contained in Syracuse watch glasses. They were left there for times which varied from 1 to 6 or 7 hours, and they were then subjected to centrifuge tests. When amoebae were carried from the culture solution to the solution of the experiment, some of the culture fluid was taken with them. Inasmuch as traces of a salt may make a decided difference in the behavior of the plasmagel, this is a real source of error. In later experiments, therefore, the amoebae were usually washed at least once in the solution whose effects were being tested, and in this way contamination with culture fluid was avoided. As a matter of fact, such washing made no difference in the case of the amoebae immersed in potassium or calcium chloride solutions, for the potassium and calcium ions appear to be especially potent. On the other hand, washing did make a very pronounced difference in the case of the amoebae exposed to pure solutions of magnesium and sodium chloride. For amoebae exposed to magnesium chloride solutions, the decrease in viscosity was somewhat greater when the amoebae were washed first and then immersed in a second bath of the solution. In the case of the amoebae exposed to sodium chloride solutions, washing is even more important. We found that when amoebae were immersed in sodium chloride solutions without preliminary washing, there was a slight increase in the centrifuge value; whereas, when amoebae were washed in the sodium chloride solution and then immersed in a second bath of the solution, there was a decrease in the centrifuge value. In discussing the effect of magnesium and sodium chloride, we shall present only the data for the washed amoebae.

Table I shows the effect of solutions of potassium chloride on the centrifuge value. The first column of the table gives the date on which the experiments were performed; the second column shows the

temperature in degrees Centigrade; the third column shows the normal centrifuge value, that is to say, the value obtained for control specimens in culture fluid; the fourth column gives the centrifuge value for amoebae immersed in m/40 potassium chloride solution; and the fifth shows the length of time the amoebae had been immersed when the definitive centrifuge value was obtained. Twenty-

TABLE I

THE ACTION OF m/40 KCl ON THE PLASMAGEL OF *Amoeba proteus*

Date	Temperature (Degrees Centigrade)	Normal Centrifuge Value	Centrifuge Value in KCl Solution	Immersion Time in Minutes
Feb. 23	21.5	80	3	278
25	22.5	80	6	300
27	22.5	80	10	251
Mar. 6	20.5	75	10	330
9	22	85	9	245
11	20	70	15	192
14	22	80	15	133
20	22.5	90	8	248
21	22.5	90	15	175
25	22.5	85	10	249
27	22.5	100	8	152
April 6	19	95	15	64
7	19.5	90	20	95
8	20	95	10	83
11	24.5	80	3	75
13	21.5	85	10	192
17	22	105	10	89
20	25	95	10	245
24	20.5	110	15	124
29	21	95	15	113
May 2	21	110	10	126
6	24.8	100	3	418
12	19	105	10	201

three experiments are listed. In the last seven experiments, the amoebae were washed in the solution and then immersed in a second bath of it. In the other sixteen experiments, the amoebae were placed directly into the solution. In every case there is a pronounced decrease in the centrifuge value.

When the values in Table I are averaged, it is seen that the centrifuge value for the amoebae immersed in potassium chloride solution is only 11.5 per cent of the centrifuge value of the normal amoebae. Thus the effect of the potassium is to cut down the centrifuge value to an eighth or a ninth of its original magnitude.

Potassium chloride, therefore, has a marked liquefying action on the plasmagel. The effect of the potassium chloride is due to the potassium ion. Potassium bromide, sulphate, and nitrate act in the same general way as does the chloride. There are minor differences in the effects of salts with different anion, but these are not great.

It is interesting to note that the liquefying effect of potassium on the plasmagel is associated with a complete stoppage of amoeboid movement. In the potassium solutions, the pseudopodia are withdrawn and there is not a trace of protoplasmic movement in any part of the cell. However, the amoeba is not killed by exposure to potassium, and it can recover its power of movement if returned to culture fluid.

TABLE II

THE ACTION OF m/60 $MgCl_2$ ON THE PLASMAGEL OF *Amoeba proteus*

Date	Temperature (Degrees Centigrade)	Normal Centrifuge Value	Centrifuge Value in $MgCl_2$ Solution	Immersion Time in Minutes
April 13	21.5	85	45	84
14	21	100	70	343
17	21.5	100	65	358
18	22	105	65	344
20	25	95	70	322
21	22.5	105	60	387
22	21	105	80	309
27	21.5	105	90	303
28	20	105	85	81
May 1	21.5	105	65	250
4	22	105	60	267
5	22.8	95	65	295

The magnesium ion acts in somewhat the same general way as the potassium ion, but it is not nearly as potent. Table II shows the effect of an m/60 magnesium chloride solution on the plasmagel of amoebae. In every case the amoebae were first washed in one Syracuse watch glass containing the solution and were then placed in a second watch glass containing a second lot of the solution. The columns in Table II are arranged in the same way as those in Table I. It will be noted that in every experiment there is a significant drop in the centrifuge value for the plasmagel of the amoebae exposed to magnesium, but this effect is by no means as great as that produced by the potassium ion.

Whereas potassium chloride solutions decrease the centrifuge value to 11.5 per cent of its original magnitude, magnesium chloride solutions decrease the centrifuge value to 68 per cent of the normal value. This figure, 68 per cent, is obtained by averaging data from the twelve experiments listed in Table II. The general action of the magnesium ion is in the same direction as that of the potassium ion, but it is not nearly as pronounced. In the magnesium chloride solutions, the amoebae are rendered practically motionless. At first sight, there is apparently no movement at all. However, if the amoebae are observed for minutes at a time, slight changes in form can usually be

TABLE III

THE ACTION OF m/40 NaCl ON THE PLASMAGEL OF *Amoeba proteus*

Date	Temperature (Degrees Centigrade)	Normal Centrifuge Value	Centrifuge Value in NaCl Solution	Immersion Time in Minutes
April 18	20.8	105	80	135
19	23	85	75	231
22	21	105	95	227
24	20.5	110	85	355
May 1	21.5	105	85	169
2	21	110	95	103
4	22	105	95	226
5	22.8	95	85	300
6	24.8	100	90	453
7	23	100	85	204
11	23	105	95	143

detected. It is interesting to note that in *Amoeba dubia* movement is not greatly retarded by dilute solutions of magnesium chloride. It is not difficult to understand the difference between the two types of amoebae. In the proteus form, the action of the magnesium ion is barely sufficient to stop movement. *Amoeba dubia* may be only slightly more resistant to the magnesium ion, but this difference is enough so that movement continues at an easily observable rate. Magnesium, like potassium, does not produce an irreversible effect, and the amoebae regain their normal power of movement when returned to the culture fluid.

Sodium chloride is still less potent than either potassium or magnesium chloride. Table III shows the results of experiments in which the amoebae were immersed in m/40 solutions of sodium chloride.

In every case the amoebae were subjected to a preliminary washing in the solution. Curiously enough, when amoebae were immersed directly into sodium chloride solutions without preliminary washing, there was a slight increase in the centrifuge value. This is a point of some interest. But for the present we shall be content with the data for washed amoebae.

In sodium chloride solutions there is only a slight lowering of the centrifugal value of the plasmagel. The data in Table III show that the centrifuge value is decreased from an average of 102.3 to an aver-

TABLE IV

THE ACTION OF m/60 $CaCl_2$ ON THE PLASMAGEL OF *Amoeba proteus*

Date	Temperature (Degrees Centigrade)	Normal Centrifuge Value	Centrifuge Value in $CaCl_2$ Solution	Immersion Time in Minutes
Feb. 25	22.5	80	130	258
27	22.5	80	130	180
Mar. 6	20.5	75	125	217
9	22	85	125	191
14	22	80	130	337
16	20	75	125	108
20	22.5	90	125	216
23	19	85	125	85
25	22.3	85	125	190
April 6	19	95	130	145
7	19.5	90	125	136
14	21	100	130	151
24	20.5	110	130	284
May 11	23	105	125	405

age of 87.7. It thus becomes 85.8 per cent of its original value. In sodium chloride solutions, the amoebae continue to move, and this movement is readily perceptible.

From the data thus far presented, it is apparent that potassium, magnesium, and sodium all lower the centrifuge value for the plasmagel of *Amoeba proteus*. Inasmuch as calcium is the only other common cation, it is fairly obvious that calcium must increase the centrifuge value. The effect of calcium is easily demonstrated. The results of a series of experiments are shown in Table IV. In every case, calcium produces a very sharp increase in the centrifugal value. It seems rather noteworthy that no matter what the normal value of a given culture may be, when the amoebae are immersed in the calcium solutions, the value always rises to approximately the same

point. In every one of fourteen series of tests, it was found to be either 125 or 130.

The data show an increase of 44 per cent in the centrifuge value for the amoebae immersed in calcium chloride solutions. The percentage increase varies from culture to culture; it is greater in those cultures which have a lower centrifugal value normally. Why there should be more variation in the centrifugal value for the amoebae in culture fluid than for those exposed to calcium chloride is uncertain. Perhaps there is a variation in the salt content of the cultures, or there may be a variation in the calcium reserve of the different lots of amoebae.

The amoebae continue to move actively in pure solutions of calcium chloride. It was noted previously that exposure to solutions of potassium chloride produced a rounding-up of the amoebae. The calcium ion appears to have the opposite effect, and amoebae exposed to calcium solutions typically become very narrow and elongate. However, there are exceptions to this rule, and some of the amoebae in the calcium solutions may look quite like those in the normal culture fluid.

Of the common cations found in living substance, calcium seems to be the only one that causes an increased stiffening of the plasmagel. Potassium, magnesium, and sodium all have the opposite effect. As far as the plasmagel is concerned, there appears to be an antagonism between calcium on the one hand and potassium, magnesium, and sodium on the other. In order to study this antagonism, amoebae were exposed to all possible combinations of these four common cations. Not all of these experiments will be reported. In general, they bear out what has already been shown, i.e., that calcium increases the centrifugal value and that the other cations decrease it. They show, too, that traces of calcium may have a very pronounced effect.

Table V shows the results of one experiment on combinations of potassium and calcium chloride. The centrifuge value was determined for mixtures of varying proportions. As before, the time which had elapsed when the definitive value was obtained is shown in the last column.

It is obvious from Table V that the action of potassium on the plasmagel is antagonized by calcium. Even 1 part of the calcium

chloride solution to a 1,000 parts of the potassium chloride solution is enough to raise the centrifuge value from 20 to 35. And in a mixture of 1 part calcium solution and 99 parts of potassium solution, the effect of the potassium ion is more than offset, and the amoebae have a centrifuge value above the normal. Similar results were obtained in other experiments. It is clear that calcium, even in small concentration, may have a very pronounced stiffening effect on the plasmagel.

Magnesium may also counteract the action of potassium to some extent, but its effect is relatively weak and it appears to be some-

TABLE V

THE ACTION OF MIXTURES OF $CaCl_2$ AND KCl ON THE PLASMAGEL OF *Amoeba proteus*

Temperature, 19.5°. Normal centrifuge value, 90

Parts of m/60 $CaCl_2$	Parts of m/40 KCl	Centrifuge Value	Immersion Time in Minutes
1	0	125	125
1	1	105	123
1	2	105	127
1	4	105	170
1	9	95	186
1	99	95	214
1	500	50	140
1	1,000	35	165
0	1	20	95

what variable. Sometimes larger quantities of magnesium seem to have less effect than smaller amounts. Table VI lists two sets of experiments. The results are not completely consistent, but they show clearly that the magnesium ion does not have the powerful effect of the calcium ion in antagonizing potassium.

The experiments in Table VI show that the magnesium ion does have an effect in neutralizing the action of potassium but that this effect is very slight compared to that of calcium. Thus, by comparison with Table V, it can be seen that 1 per cent of calcium is far more effective than 98 per cent magnesium.

Sodium also is a relatively inefficient antagonist of potassium in bringing up the centrifuge value. This is shown by the experiment listed in Table VII. In the foregoing experiment, the amoebae were

not washed in preliminary baths of the solutions. This accounts for the slight rise in the centrifuge value for the amoebae immersed in

TABLE VI

THE ACTION OF MIXTURES OF $MgCl_2$ AND KCl ON THE PLASMAGEL OF *Amoeba proteus*

Experiment 1. Temperature, 22°. Normal centrifuge value, 105.

Parts of m/40 KCl	Parts of m/60 $MgCl_2$	Centrifuge Value	Immersion Time in Minutes
1	0	10	89
9	1	40	102
1	1	15	222
1	3	40	279
1	9	15	318
1	49	20	278
0	1	65	344

Experiment 2. Temperature, 25°. Normal centrifuge value, 95.

Parts of m/40 KCl	Parts of m/60 $MgCl_2$	Centrifuge Value	Immersion Time in Minutes
1	0	10	245
9	1	40	206
1	1	30	146
1	3	35	208
1	9	25	219
1	49	60	200
0	1	70	322

TABLE VII

THE ACTION OF MIXTURES OF NaCl AND KCl ON THE PLASMAGEL OF *Amoeba proteus*

Temperature, 20°. Normal centrifuge value, 95.

Parts of m/40 KCl	Parts of m/40 NaCl	Centrifuge Value	Immersion Time in Minutes
1	0	10	83
1	1	10	142
1	3	25	190
1	9	30	186
1	49	55	222
0	1	100	216

pure sodium chloride solution as compared with the normal. The results show that sodium can antagonize the effect of potassium, but again the action of the sodium ion is vastly weaker than that of the calcium ion. In this case too, 98 per cent of sodium is less efficient as an antagonist than 1 per cent of calcium.

It might perhaps be fair to state that calcium is the only real antagonist of the potassium ion. The effects of sodium and magnesium in neutralizing the action of potassium are due perhaps as much as anything else to the dilution suffered by the potassium when it is mixed with salts of the other cations.

TABLE VIII

THE ACTION OF MIXTURES OF $CaCl_2$ AND $MgCl_2$ ON THE PLASMAGEL OF *Amoeba proteus*

Temperature, 21°. Normal centrifuge value, 100.

Parts of m/60 $CaCl_2$	Parts of m/60 $MgCl_2$	Centrifuge Value	Immersion Time in Minutes
1	0	130	151
1	1	125	164
1	3	110	197
1	9	105	215
1	49	90	220
1	99	90	237
0	1	75	135

The liquefying action of the magnesium ion is likewise antagonized by the calcium ion; and in this case also, small amounts of calcium have a marked effect. This is indicated by the data in Table VIII. Thus, when calcium chloride solution is present in 1 part in 10, the 9 parts of magnesium chloride solution are totally without effect, and the centrifuge value is increased rather than decreased. The calcium ion, therefore, is a strong antagonist of the magnesium ion, and it completely neutralizes the liquefying action of the magnesium ion. This antagonistic action of calcium toward magnesium is not shared to any appreciable extent by either the potassium ion or the sodium ion. The data in Table VI showed clearly enough that the addition of potassium ion to magnesium ion caused only a further lowering of the centrifuge value. Nor is the sodium ion an effective antagonist of the magnesium ion. This is indicated by the data in Table IX.

The results listed in Table IX show clearly enough that the sodium ion is not a powerful antagonist of the magnesium ion in so far as any effect on the consistency of the plasmagel is concerned. Even when equal parts of sodium chloride solution are mixed with magnesium solution, there is no effect of the sodium ion on the centrifuge value. This fact stands out clearly. Some of the figures in Table IX are rather difficult to interpret, and it is not easy to understand why mixtures of sodium and magnesium chloride solutions should have a greater effect in lowering the centrifuge value than either of the pure solutions. Further study of these mixtures might prove interesting.

TABLE IX

THE ACTION OF MIXTURES OF NaCl AND $MgCl_2$ ON THE PLASMAGEL OF *Amoeba proteus*

Temperature, 21°. Centrifuge value, 105.

Parts of m/40 NaCl	Parts of m/60 $MgCl_2$	Centrifuge Value	Immersion Time in Minutes
1	0	95	227
1	1	80	218
1	3	65	226
1	9	60	235
1	49	85	268
0	1	80	309

But the only point we wish to establish here is that sodium does not behave like calcium in antagonizing the liquefying action of the magnesium ion.

DISCUSSION

Our results have certainly shown that the potassium ion, and to a lesser extent the magnesium ion, produce a physical change in the plasmagel of *Amoeba proteus* of such a sort that the crystals are more readily shifted through it when the amoebae are centrifuged. We believe, therefore, that potassium and magnesium have a liquefying action on the cortical protoplasm of the amoeba. In the presence of these ions, the outer protoplasm of the amoeba becomes more fluid. Doubtless this liquefying action of the potassium and magnesium ions is directly related to their anesthetic action. Recent students of amoeboid movement favor the view that the movement of the amoeba

depends primarily on a pressure exerted by a cortical region or plasmagel. If this view is correct, any agent which would make the plasmagel more fluid would tend to retard amoeboid movement. Our studies have shown that the potassium ion, which has the most potent effect in making the plasmagel more fluid, is the very ion which is most powerful in preventing motion. The magnesium ion is also capable of liquefying the plasmagel, and this ion can cause an almost complete stoppage of motion. True, a slight movement persists in solutions of magnesium chloride, but this is indeed very slow and is only recognizable after close study. The liquefying action of the magnesium ion may also be correlated with its action on amoeboid movement.

It should perhaps again be pointed out that the centrifuge value, or the number of seconds required to cause a complete shifting of crystals in the plasmagel, is not necessarily an accurate measure of the viscosity—in part because one cannot be certain that the thickness of the plasmagel remains constant under different conditions. Possibly the action of calcium in increasing the centrifuge value is due to an increase in the thickness of the plasmagel layer rather than to an increase in the viscosity of a constant quantity of plasmagel. But whether calcium acts by stiffening plasmagel or by increasing its amount at the expense of the interior protoplasm does not so much matter. We can be certain, at least, that calcium is favoring gelation as opposed to solation. Possibly the protoplasm of the amoeba is in a continual balance between solation and gelation, and one or the other process may be continually occurring at the border of plasmagel and plasmasol. Now the effect of the calcium is certainly to favor the gelation process. Similarly, the action of potassium and magnesium is to favor the solation. Whether this expresses itself as a thinning of the outer cortical gel layer or as a softening or liquefaction of a sharply defined layer seems relatively immaterial. The important fact is that both potassium and magnesium do actually produce a decrease in the rigidity of the layer of gel which surrounds the inner fluid protoplasm.

The common cations thus exert two sorts of effects on amoeba protoplasm. Our work on *Amoeba dubia* (Heilbrunn and Daugherty, 1931) shows that the interior protoplasm is made more fluid by cal-

cium and magnesium and more viscous by potassium and sodium. But the stiffer cortical region or plasmagel is affected in very different fashion. For in this layer calcium has the very opposite effect. It produces a stiffening, and this action is antagonized by the three other common cations, especially by potassium and magnesium. Thus calcium and potassium have opposite effects on the interior and the cortex of the cell. It should be noted that our results for the interior protoplasm were obtained on *Amoeba dubia*, whereas the study of the plasmagel was made on *Amoeba proteus*. Although there is no certain proof, we have every reason to believe that the interior protoplasm of *Amoeba proteus* behaves in the same way as the protoplasm of *Amoeba dubia*. In various types of plant and animal cells, calcium and magnesium ions cause liquefaction and potassium and sodium ions produce an increase in the viscosity of the protoplasm (see Heilbrunn, 1928, chap. ix). In view of this general agreement in widely different types of protoplasm, we are no doubt safe in assuming that the interior protoplasm of *Amoeba proteus* behaves like the interior protoplasm of a form as closely related as *Amoeba dubia*. In all probability, moreover, there is a delicate plasmagel in *Amoeba dubia*, and this presumably has the same properties as the plasmagel of *Amoeba proteus*. The plasmagel in *dubia* cannot easily be studied by the centrifuge method. This may be due to the fact that it is relatively thin, or it may be due to the much larger size of the crystals in *dubia*. When *proteus* is centrifuged, many of its small crystals are completely lodged in the plasmagel. But in *dubia*, the much larger crystals of this species could scarcely be completely imbedded in a narrow gel layer. Even those near the surface of the cell would doubtless project into the central protoplasm, and it is perhaps for this reason that they are readily shifted when the amoeba is centrifuged at slow speeds. However we interpret the difference in behavior between the two species, there is good reason to believe that our results hold true for amoeba protoplasm generally, and that the interior main mass of the cell is liquefied by calcium and magnesium and made more viscous by potassium and sodium, whereas the outer cortex is stiffened by calcium and not by any one of the other cations.

It is interesting to know that the stiffer cortical protoplasm of the amoeba cell behaves differently toward reagents from the inner pro-

toplasm. It is quite possible that in many types of cells there are two types of protoplasm—a fluid mass, and a relatively stiffer material which may surround it or which may be otherwise arranged—and that these two types of material show very different behavior toward changes in the external environment of the cell. There are indications of this in the literature. And of course, there is real evidence that protoplasm may change from sol to gel and back again (see, for example, Heilbrunn, 1920). Perhaps, indeed, this is one of the most fundamental of its properties. This point of view has been emphasized in a recent monograph (Heilbrunn, 1928). The actual nature of the gelation which occurs in protoplasm is not very well understood; but there is abundant evidence that it is associated in some way with a reaction or series of reactions which, at least in the early stages, involve calcium.

It is therefore of particular interest to note that the calcium ion favors gelation or increased stiffening in the outer cortex of the amoeba, and this is a fact which is in thorough harmony with the point of view of the concluding chapters of the monograph to which we have just referred. This point of view holds that protoplasmic activity is dependent on a coagulation or gelation process in the protoplasm and that this gelation is fundamentally what is termed a "surface precipitation reaction." The surface precipitation reaction is that series of reactions which occurs when a cell is torn. It may also occur within intact cells; and when it does, it always produces a sharp increase in viscosity. Concerning the intimate nature of the surface precipitation reaction, little is known; but in many ways it resembles blood clotting, and, like blood clotting it depends in its first stages on the presence of calcium.

For some time, it has been known that amoeboid movement is dependent on the presence of calcium (Pantin, 1926; Pollack, 1928). Now it appears that calcium actually favors a gelation process in the outer rim of the protoplasm. The anesthetic action of the potassium and magnesium ions is associated with an anti-gelatinizing effect of these ions. Thus, in the amoeba, activity is associated with gelation, and anesthesia or prevention of activity is associated with prevention of gelation. Moreover, the gelation in the cortical protoplasm of the amoeba is certainly bound up in some way with the presence

of the calcium ion. These facts are surely in excellent agreement with the theory of protoplasmic activity as previously outlined.

However, when we search for an explanation of why it is that the potassium ion can have a liquefying action, we are at a loss. This is certainly an important question and one worthy of further study.

The results of this investigation should offer some clues toward an understanding of the antagonistic action of the common cations of protoplasm. The whole question of antagonism is a difficult and involved one. For in various biological processes, different antagonisms may occur. In one case, two ions may be opposed to each other; whereas in another instance, the same ions may be acting together. From the work on amoeba, it is clear how such differences may arise. The effects of the ions may be on different regions of the cell or on different constituents of the protoplasm. Thus calcium and magnesium act together in producing liquefaction in the cell interior, but they have opposite effects on the cortex.

It is to be hoped that a study of the action of various cations on the protoplasm of the cell may eventually lead to a solution of some of the difficult problems of antagonism in various complicated living systems.

SUMMARY

1. When *Amoeba proteus* is centrifuged, the crystals in its interior are readily moved by small centrifugal forces operating for short periods of time. But the protoplasm in the cortex of the cell, the plasmagel, is much stiffer, and the crystals in it are not shifted by centrifuging unless strong force is used for a considerable time interval.

2. When the amoebae are centrifuged at high speeds, the time necessary to produce a definite degree of shifting of crystals in the plasmagel is regarded as a measure of the stiffness or degree of gelation of this cortical region of the amoeba protoplasm. The time in seconds is called the "centrifuge value."

3. The calcium ion markedly increases the centrifuge value of the plasmagel. Potassium, magnesium, and sodium decrease the centrifuge value.

4. The effect of potassium is particularly pronounced, and in

potassium solutions the centrifuge value is reduced to about an eighth of its normal magnitude. This effect of potassium is correlated with a complete stoppage of movement.

5. Magnesium also causes a drop in the centrifuge value, but the effect of magnesium is not as great as that of potassium. Solutions of magnesium chloride stop movement, but not completely.

6. Sodium has only a slight effect. It produces a small drop in the centrifuge value. The amoebae continue to move in solutions of sodium chloride.

7. The results of the experiments are of interest for the theory of the antagonistic action of salts. They also have a very direct bearing on the general theory of protoplasmic activity. Something of this is brought out in the discussion.

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GROWTH AND RESORPTION PHENOMENA IN ANURAN METAMORPHOSIS. I

A. Time Relationships in Metamorphic Incidents
B. Growth of the Legs in Length
C. Desiccation Phenomena
D. Changes in Body Weight

(Five figures)

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INTRODUCTION

AN EXPERIMENTAL analysis of any phenomenon presupposes an exact and complete description of it. Yet in the study of amphibian metamorphosis much labor has been expended in experimental work without an adequate descriptive basis. This deficiency has recently met recognition in the studies of Wilder (1925) and of Grant (1930), who have worked out the various incidents of urodele metamorphosis in relation to time and to each other. On the other hand, I fail to find in the literature of anuran growth and metamorphosis an exact study of the sequence of the metamorphic incidents, although many valuable descriptions and notes are available, scattered throughout the literature.

That such a study, especially if undertaken from the quantitative aspect, would help to elucidate some points and suggest many avenues of attack upon the problems of metamorphosis seems very likely, and this investigation was therefore undertaken with this end in view.

The period of metamorphosis in Anura is here understood to include the period of development of the hind legs from their buds, a usage which conforms to that of the majority of experimental workers on Anura but differs from that of many naturalists and older workers, as Boulenger (1897) and Schaper (1902), who restrict this term to the period of tail resorption, mouth changes, etc. This later period will be termed the "metamorphic climax."

A. TIME RELATIONSHIPS IN METAMORPHIC INCIDENTS

In this section data on the time relationship and variability of the incidents of metamorphosis will be presented. From these an attempt will be made to present a picture of "normal" metamorphosis in the three species of *Rana* employed—*R. palustris*, the pickerel frog; *R. clamitans*, the green frog; and *R. catesbeiana*, the bullfrog. Such a description of the metamorphic events will furnish the basis for seriating tadpoles with regard to the progress of metamorphosis.

MATERIAL AND METHODS

In the summer of 1930 tadpoles of *Rana palustris* were observed. These were all derived from one batch of eggs laid in captivity by a pair of adults caught during amplexation. The eggs were laid on May 1. The tadpoles were reared in captivity in battery jars 6 by 8 inches and fed on a diet of algae growing in the jars, and cooked and ground liver that was added daily.

About a week before observations were begun, the animals were each placed in individual jars. The jars were then placed in a large copper tank on an open veranda, where they received 2–3 hours sunlight each morning. In order to keep the temperature more uniform, and especially to prevent overheating due to sunlight, the tank was kept filled with water so that the jars were always in a water bath. The water in the jars was changed daily. The food was the same as previously.

At first no special attempt was made to keep the temperature constant, but fortunately during the major part of the period of measurement (late July and early August) the weather was unusually constant and very warm. However, while some few tadpoles were in the last stages of this process, a cold spell suddenly appeared on August 11. After the thirteenth of August the temperature of the water was kept up to between 25° and 30° C. (approximately its former temperature) by introducing electric bulbs into the water bath. That the drop in temperature had a decided effect upon the growth of the tadpoles will be shown below.

Fortunately, the drop in temperature caught all but three of the animals in the last stages of metamorphosis and was uncontrolled for only a short time, so that the variation in the figures can be

ascribed to it only in small part. During the resorption of the tail, the animals were placed in such a position that they could choose either a water or air medium, and were kept indoors.

That this treatment was not entirely successful in imitating natural conditions is seen in the fact that about one-half of the animals showed some abnormality to a slight degree, such as the kinking of the tail or imperfect foot development. Yet, even in severely affected cases, these abnormalities had no apparent effect upon the metamorphic process.

In the summer of 1931 the observations were repeated on *Rana clamitans* and *R. catesbeiana*. In this work the tadpoles were collected in the premetamorphic stage at ponds near Alpine, New Jersey. All the specimens of one species came from the same pond and were collected in late March or early April. The treatment of the animals was essentially the same as in the previous summer except that before May 3 they were kept indoors. Complete sets of records are available for 11 specimens of *R. catesbeiana* and for 14 of *R. clamitans*.

RESULTS—RANA PALUSTRIS

The daily observations on 20 specimens and summaries are presented in Table I. As the chief object of the study was to determine the time relation of metamorphic incidents, tabulation of the actual age in days from laying or hatching to the occurrence of the various changes was considered of little value, owing to variations in rate of growth of different tadpoles even under uniform conditions. Therefore, the most definitely determined event, the emergence of the forelegs through the operculum, was taken as a basis for timing all of the other metamorphic incidents. The emergence of the forelegs was arbitrarily set at the 30th day, and the other events are recorded according to their time relation in days to this event. The days given in the table are therefore relative.

In Table I, column 1 gives the day on which the hind legs reached a length of 5 mm. This was determined by interpolation in the growth curves of the hind legs of the specimen. The time was found to vary from the 9th to the 13th days in the 13 specimens available, with the mode at the 10th day.

The time of the first definite appearance of the dorso-lateral light

lines is recorded in column 2. These lines form in the position of the dorsal plicae, but the relation of the pigment change to gland formation has not yet been determined.[1] The day given is that of the first

TABLE I

TIME RELATION OF METAMORPHIC INCIDENTS

The emergence of the forelegs is arbitrarily set at the 30th day, and all other events are timed in days from this. An. No. = number of animal; H. L. 5 Mm. = time when hind legs reach length of 5 mm.; D. L. L. L. = first appearance of dorso-lateral light lines; D. L. S. R. = first appearance of dorso-lateral spot rows; S. W. F. L. = skin-window for the forelegs; Em. F. L. = emergence of forelegs; Ch. B. = loss of the chitinous beak; Eyelid = appearance of the eyelid; Skin Shed = first appearance of particles of shed skin in the water; Res. Tail = resorption of the tail definitely visible; Gill S. W. = formation of gill-skin-window; Fr. M. Comp. = frog mouth completely formed; Oper. Clos. = opercular cavity closed; Tym. = tympanum formed.

An. No.	1 H. L. 5 Mm.	2 D.L. L. L.	3 D.L. S. R.	4 S. W. F. L.	5 Em. F. L.	6 Ch. B.	7 Eye-lid	8 Skin Shed	9 Res. Tail	10 Gill S. W.	11 Fr. M. Comp.	12 Oper. Clos.	13 Tym.	14 H. L. Length at Em. F. L.
3	10	.....	.....	.....	30	30	30	.....	31	.....	33	34	37	37.5
4	10	19	22	27	30	31	31	30	31	32	.....	.....	.....	41.2
5	.....	24	27	28	30	30	.....	.....	.....	31	.....	.....	.....	37.9
6	14	.....	.....	.....	30	30	31	.....	31	.....	33	.....	.....	39.0
7	10	20	26	27	30	30	31	31	31	31	34	.....	.....	35.5
8	12	23	25	28	30	30	32	33	33	32	34	37	39	33.9
9	13	22	23	28	30	30	31	33	32	32	34	36	.....	38.0
10	12	21	23	28	30	29	30	32	32	33	33	36	38	34.0
11	13	24	26	29	30	30	31	37	32	.....	33	36	38	.....
14	9	20	20	28	30	30	31	31	31	30	.....	.....	.....	31.5
15	13	22	24	27	30	29	32	32	32	31	33	36	38	33.0
17	10	21	21	27	30	31	31	31	32	31	.....	.....	.....	32.0
18	10	19	21	28	30	31	31	30	32	31	.....	.....	.....	34.0
19	12	19	20	27	30	30	30	30	.....	30	.....	.....	.....	39.0
20	.....	.....	26	27	30	30	30	.....	31	30	34	36	38	31.0
21	.....	.....	27	.....	30	31	.....	.....	32	.....	34	34	37	.....
23	.....	.....	27	.....	30	.....	31	.....	31	.....	34	35	37	.....
24	.....	.....	26	.....	30	30	.....	.....	32	.....	33	36	37	.....
26	.....	21	22	27	30	31	31	31	32	31	34	.....	.....	.....
27	.....	.....	27	29	30	30	32	.....	31	.....	33	36	37	.....
Range	9–14	19–24	20–27	27–29	30	29–31	30–32	30–37	31–33	30–33	33–34	34–37	37–39	31–41.2
Mode	10	19–21	26–27	27	30	30	31	31	32	31	33–34	36	37	.....
No. of animals	13	13	18	15	20	19	17	12	18	13	14	11	10	14

definite and unquestionable appearance of the light lines. The time varies from 19 to 24 days in the 13 specimens, with the greatest frequencies at the 19th and 21st days.

The third column gives the day of the first evidence of the characteristic large pigment blotches of the adult. Those studied were the two rows on the back between the dorso-lateral light lines. Again only the unequivocal first record was accepted. First appearance of

[1] Recently Helff (1931) has given evidence that they are simultaneous.

the pigment blotches varied from the 20th to the 27th day in 18 specimens.

In the fourth column the day of the first appearance of the skin-window for the forelegs is recorded. At the posterior edge of the operculum the skin disintegrates and its white pigmentation disappears, with the resulting formation of a thin transparent area of skin through which the forelegs break. The formation of the skin-window is a rather rapid process and occurred on the 27th to the 29th day in 15 specimens.

The emergence of the forelegs (column 5), as already explained, was assumed to occur on the 30th day. In all cases observed, both legs emerged on the same day. The day of the loss of the chitinous beak is recorded in column 6 and is seen to vary from 29 to 31 days in 19 specimens. By "the first appearance of eyelids" (column 7) is meant the first day upon which the lower lid may be lifted up so as to at least half close the eye. In the 17 specimens recorded this took place on the 30th to the 32d day. It is at this time that the eyes undergo the major part of their remarkable growth.

The first time particles of shed skin were found in the water in which animals were kept is recorded in the eighth column, and varied from the 30th to the 33d day, except in specimen 11, where the time is placed at 37 days. The possibility of overlooking small pieces of skin among the algae may account for this late record. In the ninth column the first day on which tail resorption was obvious is noted. Since this phenomenon proceeds very rapidly, the tail usually being reduced to a stub in 3 days, no great error is introduced by basing the records on a stage when the tail reduction was obvious and could be readily diagnosed. In 17 specimens the time recorded was the 31st or 32d day; in one, the 33d day.

In the tenth column the time of the first record of the formation of a skin-window in the region of the operculum just ventral to the gills is noted. This area of degeneration spreads rapidly, forming an extensive bib that is broad ventro-laterally and extends dorsally as a thin area to the mid-dorsal line from each side. This skin-window is strikingly similar in appearance to that formed for the emergence of the forelegs but, so far as observed, had no particular function, nor did it rupture. It later underwent repair. This formation is distinct

in both time and place from the skin-window for the forelegs, for it first appears on the 30th to the 33d day (13 specimens) in a region anterior to, and more extensive than, the latter. As its height this window outlines the gill cavity and is hence termed the "gill skin-window."

The changes in the mouth region at this time are very marked, a general broadening of the mouth being the most obvious. This occupies 3 days usually, and the record given in column 11 shows that in 14 cases it was completed on either the 33d or the 34th day.

On the emergence of the forelegs, an opening between the gill chamber and the outside is, of course, established; and although this closes by fusion on the posterior side of the leg base, the anterior part of the opening remains patent until the repair of the gill skin-window is completed. The closure of these openings furnishes a convenient landmark in metamorphosis and, as seen in column 12, occurred on the 34th to the 37th day in 11 specimens.

The completion of the process of metamorphosis, as I have observed it, waits upon the definition of the tympanic membrane. This is most easily recognized in its early stages by the elevation of the annulus around it. In the thirteenth column of the table, the records of 10 individuals are given in which this process is seen to occur on the 37th to 39th day.

There are, of course, many other external changes during metamorphosis. In the later part of the prometamorphic period the formation of the dorsal gland ridges, and especially the resorption of the extension of the anal canal into the tail, are noteworthy. In general, however, the events given above seemed the most easily determined ones.

DISCUSSION

The process of metamorphosis in *Rana palustris* is seen to involve a definite series of events that bear a rather constant time relationship to each other under normal conditions of growth. Figure 1 represents an attempt to give a compact summary of these events. This is prepared by marking off the daily intervals on a horizontal, and by a horizontal bar indicating the period during which each phenomenon might be expected to occur. While it must be recognized that the small number of cases upon which this table is based

precludes the consideration of it as a complete picture of the normal relations described, yet it is hoped that, when properly used, it will furnish a rough standard by which the "normality" of experimentally induced metamorphosis may be judged.

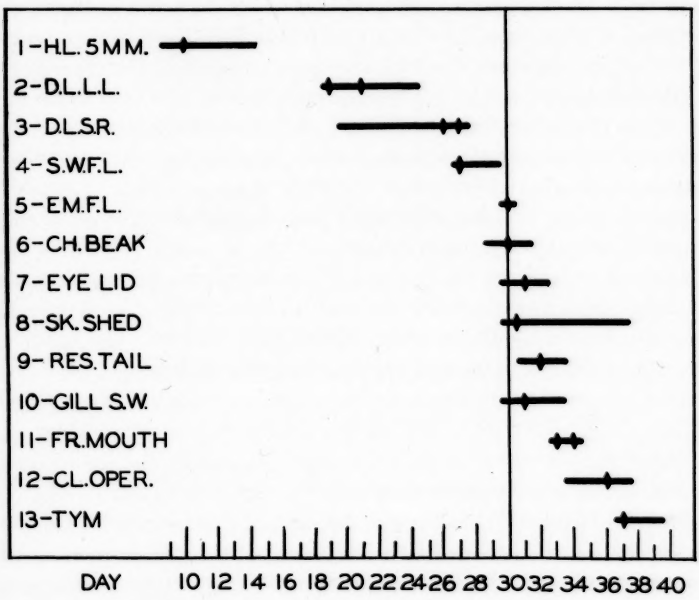

FIG. 1.—Time relationships and variability of the events of normal metamorphosis. The arrowheads indicate the mode for each process; the horizontal bar, the number of days occupied by the process for all of the specimens. H. L. = hind legs; D. L. L. L. = dorso-lateral light lines; D. L. S. R. = dorso-lateral spot rows; S. W. F. L. = skin-window for the forelegs; Em. F. L. = emergence of the forelegs; Ch. B. = loss of the chitinous beak; Eyelid = appearance of the eyelid; Sk. Shed = appearance of particles of shed skin in the water; Res. Tail = resorption of the tail; Gill S. W. = gill-skin-window; Fr. mouth = frog mouth completely formed; Oper. Cl. = opercular cavity closed; Tym. = appearance of the tympanum.

A further use to which this table can be put is that of aiding in the seriation of tadpoles. This matter deserves further consideration, as it will be referred to repeatedly in the course of this work. The difficulty of definitely and accurately expressing the degree of development of a given tadpole with respect to metamorphosis has ham-

pered the analysis of this phenomenon. Age of the animals is utterly useless, since the age variation at which metamorphosis takes place even in a carefully controlled stock is often greater than the entire duration of the process. The most widely used criterion has been the degree of development of the hind legs. As will be seen in the second section of this paper, this is adequate during the earlier period of metamorphosis when the legs are growing rapidly. But during the metamorphic crisis, the growth is much slower, and, as can be seen in the fourteenth column of Table I, the variation in size of the hind legs at the time of tail resorption is too great to permit its use where precise timing is desired. It is therefore suggested that tadpoles be seriated during the metamorphic climax by the occurrence or non-occurrence of the events tabulated.

A term to indicate the period of metamorphosis before the metamorphic climax would be convenient. I therefore have used the term "prometamorphosis" to cover the period between the onset of growth of the hind legs and the metamorphic climax.

RESULTS—RANA CLAMITANS AND RANA CATESBEIANA

With these results on *Rana palustris* in mind, the situation in *R. clamitans* and *R. catesbeiana* may be very briefly reviewed. The sequence of events is, in all cases, the same and corresponds essentially to that in *R. palustris*. The more important differences in the nature of the externally evident changes may be summarized as follows:

Pigmentation changes.—Definitive pigmentation changes are difficult to observe in the latter two species and have not been recorded. The dorsal lateral light line does not appear at all in the bullfrog.

Skin window for the forelegs.—In *Rana clamitans* this becomes noticeable usually only on the day before the emergence of the forelegs; but in the bullfrog tadpoles the initial appearance of the transparency becomes evident much sooner, generally in 6–10 days, and in one case 11 days before the emergence of the forelegs.

Resorption of the anal canal piece.—The anus opens from the end of a canal running in a mass of connective tissue along the ventral tailfin for a distance of about $\frac{1}{4}$ inch. During late prometamorphosis,

as the legs develop, this is resorbed and constitutes the first evidence of resorption phenomena that I have noticed.

Emergence of the forelegs.—This usually occurs in both species with the left foreleg first or both on the same day, but one case in the green frog is noted in which the right emerged first.

Resorption of the tail.—The most useful date for this is the day on which such resorption can be noted without measurement, and this has been recorded. If, however, daily records of tail length are kept, it is found that initial reduction can be noted about 2 days before it becomes obvious without measurement (see Figs. 4 and 5).

Skin shedding.—Careful observation in these specimens showed that the first skin shreds can be found in the water within 1–5 days after the emergence of the forelegs. Thereafter, occasional pieces will be found almost daily for several weeks, though they decrease quickly in amount.

Change in the respiratory medium.—It is possible to observe just when the animal changes its mode of respiration from the tadpole to the frog type by placing it in water to a depth just covering the snout in the normal sitting posture of the animal. An air breather will keep the nostrils just above the surface; a water breather will not. Although there seems to be a period of indefinite reaction, this lasts less than 1 day, and a definite change to air breathing is found to occur in the 1st or 2d day of tail resorption.

With regard to the timing of these events, the following points are noteworthy.

The length of the metamorphic period is greater in these two species than in the pickerel frog, though not in direct proportion to the sizes of the animals (see tabulation below). The greater length of time that the observations covered, as well as the difference in the time of the year in which different animals went through metamorphosis, resulted in considerable variations in temperature; and this is reflected in a large variation in the time relation of the events. Since, however, no change in the sequence of events was produced, this variation is of no special significance except to indicate the dependence of the rate of development on temperature. An example may be noted. The time from the emergence of the forelegs to the closure of the operculum in early May in two green frogs was 19 and

20 days, respectively. In late July and August this same period was passed through in 5 or 6 days. It can be seen, therefore, that a tabulation of the time required in cases that varied so in their conditions can reveal little. If, however, the specimens metamorphosing during July and August are considered, a typical seriation of the events may be approximated and can be related to the comparable table for *Rana palustris* and can be used as a basis for the seriation of tadpoles.

Such a typical picture in the two species is given below.

Rana catesbeiana Relative Day E. = 30		*Rana clamitans* Relative Day E. = 30
1	Hind legs reach 10 mm. Differentiation into the adult regions complete	7
20	Anal canal piece completely resorbed	25
23	Skin window for forelegs distinctly visible	29
30	Emergence of both forelegs	30
30	Loss of lower chitinous beak	31
31	Loss of upper chitinous beak	31
32	Eyelid $\frac{1}{2}$–$\frac{2}{3}$ developed. Eye rapidly enlarging	31
33	Flakes of shed skin appear in the water	33
34	Beginning of tail resorption	33
35	Animal breathes through nostrils regularly	34
36	Widening of mouth completed to corners of jaw	34
39	Posterior openings of opercular cavity completely closed off	35
40	Tympanum clearly defined by annulus	37

B. GROWTH OF THE LEGS IN LENGTH

1. HIND LEGS

This section reports an investigation of the rate of growth of the hind legs during metamorphosis in *Rana*. The objects to be accomplished by such a study may be briefly stated as: (1) to consider the relation of the resorption phenomena at the metamorphic climax to the growth of the legs; and (2) to furnish the basis for converting data based on hind-leg length to a time curve.

The animals used were the same tadpoles which served as material for the first section, and correspond number for number. A discussion of the origin and care of the animals need not, therefore, be repeated here.

Measurements were taken as follows. The animal was removed from the water and laid on its back on a block of varnished wood. The body length was then measured by means of vernier calipers reading to 0.1 mm., and the animal returned to the water. After about 1 minute the animal was again removed and the hind-leg length taken. In this way drying of the skin and undue interference with the growth of the tadpoles was avoided. Measurements were usually taken twice weekly, sometimes three times. The length of the hind leg was measured from the extreme tip of the fourth digit to the point of insertion of the leg into the body along the lateral surface while the leg was gently extended backward so as to straighten it. The hind legs exist as buds, apparent externally, from at least as early as the 10 mm. (body length) stage in *Rana palustris*. The measurement of these buds is difficult and, since it is not of interest here, was not extensively made. In *R. palustris*, after the buds reach a length of 5 mm. they begin to grow definitely and rapidly, and measurements were therefore initiated at the 5 mm. stage. The corresponding stage in *R. clamitans* was taken at 8 mm. and in *R. catesbeiana* at 10 mm.

RESULTS

1. *Rana palustris*.—The data on the growth of the hind legs are given in Table II for 15 animals. The data for specimen 11 are plotted in Figure 2, from which it can be seen that the curve is of sigmoid form. The period of rapid growth occurs between the lengths of 5 and 37 mm. When the curves for the various specimens are compared, they all show the same sigmoid curve; but the hind-leg length at which growth slows down exhibits some variability. The range of variability was 31–42 mm. in the various specimens, as can be seen from Table II, with the mode at about 37 mm. Below Figure 2 are letters indicating the time at which certain other definite phenomena have occurred in this specimen. These data are derived from Table I. As the relations shown here in Figure 2 are almost exactly duplicated in all of the other specimens, we may accept them as typical of the metamorphic process in this species. It is noteworthy that the sudden decline in the rate of growth of the hind legs occurs at the metamorphic crisis, and this fact is of significance in relation

to the influence of the thyroid on hind-leg growth. Rapid hind-leg growth is typical of the prometamorphic period.

One of the purposes of this study was to furnish a basis for the construction of time curves by which data compared to hind-leg length can be converted into time curves or growth curves. To accomplish this, the "abscissa rule" given below Figure 2 was constructed. This was made by carrying out the ordinates to the growth curve and dropping a perpendicular from the point of intersection to a horizontal below the abscissa line. This horizontal was then

TABLE II

GROWTH IN LENGTH OF HIND LEGS OF TADPOLES OF *Rana palustris*
(Figures in Millimeters. Eggs laid May 1)

Animal No.	July 15	July 16	July 17	July 24	July 27	July 31	Aug. 3	Aug. 7	Aug. 10	Aug. 12	Aug. 14	Aug. 17	Aug. 19
3	5	.....	.....	16.7	23.4	32.5	36.8	39.4	40.5	41.0	41.1	41.4	41.5
4	.....	.....	.....	3.7	6.7	10.5	17.0	26.9	35.4	37.3	41.2	41.8	43.5
5	.....	.....	.....	9.7	16.8	24.0	31.1	37.9	.....	.....	.....	.....	.....
6	2.3	.....	.....	20.0	30.8	37.6	42.0	.....	.....	.....	.....	.....	.....
7	.....	.....	.....	5.5	8.6	10.8	16.0	25.1	32.7	34.7	36.3	37.5	38.0
8	.....	.....	.....	6.6	9.4	13.9	19.9	29.0	33.9	35.5	36.3	36.9	38.3
9	.....	.....	.....	5.4	8.8	14.6	21.4	32.7	38.0	39.0	41.0	41.5	41.6
10	2.1	.....	.....	5.6	8.2	13.3	19.0	28.2	33.2	35.0	36.0	37.5	37.9
11	.....	2.5	.....	7.9	12.5	20.4	27.4	35.2	37.0	37.5	37.7	38.5	38.6
14	.....	.....	.....	4.0	5.9	7.7	10.4	18.1	23.9	26.7	30.0	32.6	.....
15	.....	.....	1.7	4.4	7.0	12.5	18.8	27.5	32.9	33.3	34.6	36.4	36.4
17	.....	.....	2.3	5.0	6.5	10.5	15.0	23.7	30.0	31.0	33.3	.....	.....
18	.....	.....	2.0	4.0	6.4	10.0	14.5	22.6	30.7	30.5	34.0	35.6	37.6
19	.....	.....	.....	.....	2.7	4.0	7.3	11.5	20.0	22.5	27.6	36.1	39.0
20	.....	.....	.....	6.2	9.7	13.9	19.1	27.6	32.0	33.5	34.0	34.9	.....

marked with the corresponding ordinate. Since the growth of the hind legs is rapid until about the time of emergence of the forelegs, this "abscissa rule" is satisfactory only until that point; thereafter the growth is so slow that the variation of the data in different animals makes the figures of little value. Therefore, during the metamorphic crisis the "abscissa rule" has been constructed with the aid of other data derived from Table I. These are inserted into their proper time relationship to complete the rule, thus giving the basis for seriating tadpoles during the metamorphic climax.

2. *Rana clamitans and R. catesbeiana.*—The foregoing discussion was written before the data on the green frog and the bullfrog were available. However, it may be applied in all essentials to these two

species. An extended discussion of the later results is therefore not necessary, and the points of difference may be briefly noted.

In both *Rana catesbeiana* and *R. clamitans* the growth curve is sigmoid in shape, as in *R. palustris;* but the rapidity of the growth varied according to the time of the year and the temperature. For

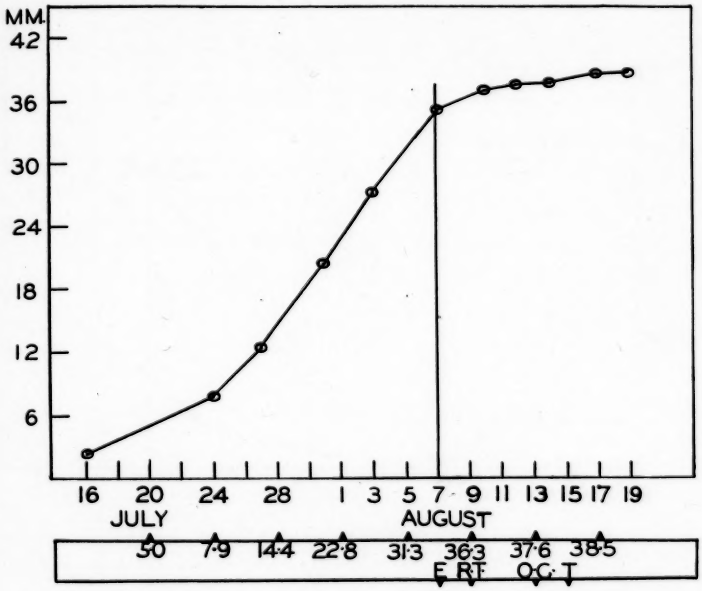

FIG. 2.—Growth curve of the hind legs during metamorphosis for specimen No. 11. Beneath the curve is the "abscissa rule" derived from the growth curve as stated in the text. E = emergence of the forelegs; R. T. = resorption of the tail; O. C. = closure of the opercular opening; T = appearance of the tympanum.

example, two specimens of the bullfrog which reached a stage of 10 mm. hind-leg length in April required 78 and 88 days, respectively, before reaching the stage of development at which the emergence of the forelegs occurred; but in four June and July specimens, 28–30 days sufficed. This is in accordance with the marked effect of the same conditions that was noted in the first section of this paper.

The time at which the plateau of growth rate is reached is somewhat later in these species. In the green frog almost all specimens

show the rate of growth slowing up decidedly between the time of foreleg emergence and tail resorption. In the bullfrog the same phenomenon is observed slightly later, between tail resorption and the appearance of the tympanum. Typical graphs of animals metamorphosing in July are shown in Figures 3 and 4. The corresponding "abscissa rules" derived as described for *Rana palustris* are given.

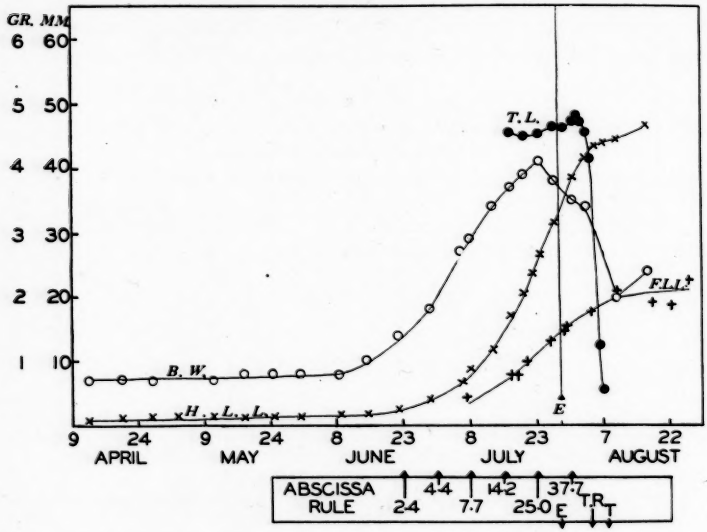

FIG. 3.—Growth curve of the body weight (B. W.), hind-leg length (H. L. L.), foreleg length (F. L. L.), and tail length (T. L.) in *Rana clamitans*. Abbreviations as in Figure 2.

2. FORELEG GROWTH

In the course of the dissections necessitated by the work on the desiccation phenomena reported below, it was found possible to dissect the forelegs out of the opercular cavity in which they develop and to measure them. This was done only for the green frog and the bullfrog.

The measurements were made by dissecting the limb free, measuring from the exposed head of the humerus to the elbow, and then from the elbow to the tip of the third finger and by adding these

lengths. Two measurements were necessitated by the fact that the partly developed limb is firmly flexed at the elbow. The result of this study is shown in Figures 3 and 4. This curve is a growth curve comparable in nature to that of the curve of the hind-leg length. In

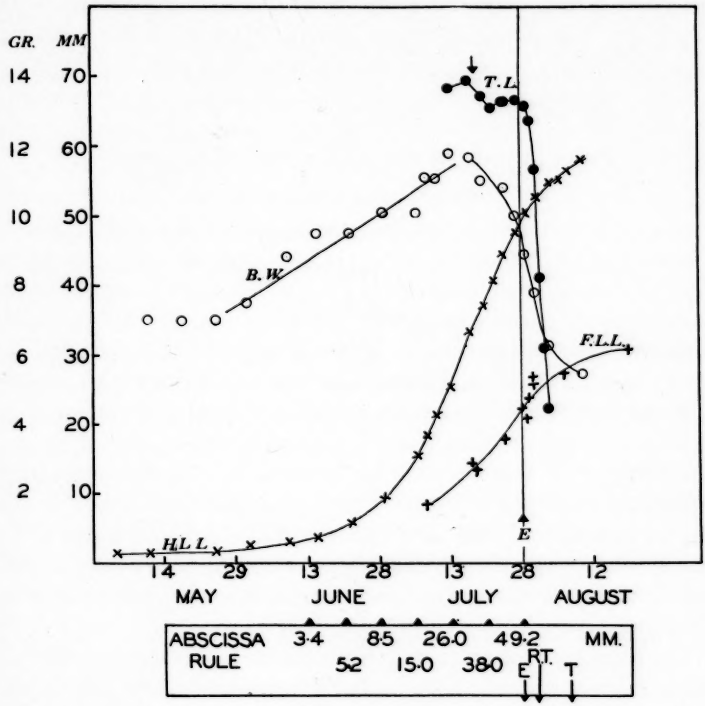

FIG. 4.—*Rana catesbeiana*. Arranged as Figure 3. The arrow on the curve for tail length indicates the day in which the tip of the tail was lost accidentally.

order to throw the data into this form, it was necessary to use as the abscissa, not the original abscissa of the graph, but rather the abscissa rule given below it.

It is apparent from the figures that the shape of the growth curve of the forelegs is the same as that of the hind legs but that the forelegs lag behind the hind legs in their growth. This same lag was apparent in the dissections of younger animals where it was found

that the differentiation of the forelegs takes place later than that of the hind legs.

A discussion is deferred until the succeeding section. From the data presented here the following conclusions may be drawn: (1) The growth in length of the legs is seen to fit on a typical sigmoid growth curve. (2) The period of rapid growth in length is the prometamorphic period. (3) The metamorphic crisis is a period of considerable slowing-down of the rate of growth of the legs. (4) It is clearly evident that the resorption of larval organs during the metamorphic climax does not furnish the material for the building of the legs.

C. DESICCATION PHENOMENA[1]

That there is a pronounced loss in weight in the metamorphosing tadpole has been known for many years. As early as 1902, Schaper has made an exact study of this in *Rana esculenta* and reported a loss of over 50 per cent. This author and many others after him have suggested that this decrease in weight is largely to be accounted for by the desiccation of the tissues. The validity of this explanation is supported by the fact that the ratio of dry to total weight more than doubles during metamorphosis, as shown by Schaper (1902) and Etkin (1930). I felt that this important aspect of metamorphosis deserved a more precise investigation, especially with regard to the rate of desiccation. The data collected on this point concern the ratio of dry to net weight at different times in the developmental period, and from these data a curve showing the rate of desiccation is derived.

The tadpoles used in this study came from the same sources as those employed above, and received the same general treatment, except that they were kept in a well-lighted room rather than outdoors and that a number of animals, varying from 20 at first to 6 or less during metamorphosis, were kept in the same jar.

The technique of determining the dry weights of the animals was as follows: The animal selected was usually of about the mean degree of development of the batch. It was weighed and measured (according to the technique of measurement described in the second section). This weight in the living state was termed the "gross

[1] I am indebted to Professor C. R. Stockard for permission to use the equipment of the department of anatomy at Cornell University Medical College in this work.

weight." The tadpole was then placed in the dried and tared crucible, the head and tail cut off, and the body wall opened. The intestine was then removed entire, drained into the crucible, and placed in a watch glass of water. The intestinal contents were then eliminated by cutting the intestines into strips about 2 inches in length, placing these separately on a piece of paper, and pressing out the contents with a needle. The pieces of intestine were then returned to the crucible after excess fluid was drained off, and the whole weighed again. This weight was called the net weight. The crucible was next put into a gas drying-oven kept at 95°–100° C. and allowed to remain there until the contents were thoroughly dry. In the smaller animals this was accomplished in 5–6 hours, as determined by repeated weighings and dryings. As larger animals require at least 18–24 hours, even when cut into several pieces, these were generally left 3–4 days and then weighed only once.

The percentage of dry weight was calculated as dry weight over net weight rather than as over gross weight, since in the later stages of metamorphosis the intestinal contents are entirely eliminated owing to the cessation of feeding.

In the determination of the ash, several animals were introduced into the crucible and, after the treatment described above, were incinerated over a Fisher burner until reduced to a white powder. In the smaller stages, 5 animals were used for each determination, but later this was reduced to 2, and finally 1.

RESULTS

1. RANA PALUSTRIS

Forty-two determinations of dry weight and eight of ash weight were made. These data are given in Tables III and IV. From these data it can be seen that during tadpole life the percentage of dry weight rises from slightly less than 6 to about 9 per cent; during metamorphosis it rises from about 9 to about 21 per cent. The percentage of ash shows a similar relation, but the data are not sufficiently extensive for detailed analysis.

A more adequate idea of the rate of desiccation in relation to anuran development can be obtained from the curve presented in Figure 5. This has been constructed with the aid of the "abscissa rule" de-

TABLE III

NET WEIGHT AND DRY WEIGHT OF TADPOLES OF *Rana palustris* AT DIFFERENT BODY AND HIND-LEG LENGTHS

Specimen No. Series M.	Body Length (Millimeters)	Hind-Leg Length (Millimeters)	Net Weight (Grams)	Dry Weight (Grams)	Ratio Dry to Net (per Cent)	Notes
1	6.1	Bud	0.0348	0.0021	6.0	
2	7.3	Bud	.0532	.0030	5.6	
3	6.5	Bud	.0392	.0022	5.6	
4	6.8	Bud	.0572	.0032	5.6	
5	6.8	Bud	.0494	.0031	6.3	
6	10.6	Bud	.1464	.0100	6.8	
7	10.6	Bud	.1714	.0117	6.8	
8	11.0	Bud	.1776	.0129	7.3	
9	11.4	Bud	.2148	.0139	6.5	
10	11.2	Bud	.2112	.0140	6.6	
11	16.4	Bud	.5096	.0436	8.6	
12	14.6	Bud	.3860	.0334	8.7	
14	14.6	Bud	.3782	.0332	8.8	
15	14.7	Bud	.4236	.0320	7.6	
16	15.9	Bud	.5453	.0437	8.0	
19	19.0	Bud	.9010	.0795	8.8	
20	19.6	Bud	0.9189	.0781	8.5	
21	19.1	Bud	1.0486	.0940	9.0	
23	21.3	6.6	1.4120	.1414	10.0	
24	21.0	15.4	1.5982	.1908	11.9	
25	19.4	7.5	0.9968	.0924	9.2	
26	20.5	10.8	1.4363	.1514	10.5	
27	22.5	21.6	1.9893	.2437	12.3	
28	23.9	17.2	1.9351	.2349	12.1	
30A	18.6	4.1	0.9334	.0918	9.8	
31	22.3	11.5	1.7736	.1950	11.0	
32	24.0	20.1	2.4020	.3228	13.4	
33	22.5	36.5	1.8603	.3401	18.3	Tail resorption
34	21.4	34.3	2.6180	.4246	16.2	Forelegs out 1 day
35	21.9	30.3	2.3880	.3287	13.8	Forelegs about to emerge
36	23.4	16.5	2.0418	.2506	12.3	
37	23.2	36.5	2.9902	.4484	14.9	Forelegs about to emerge
39	25.5	24.5*	1.6412	.3386	20.6	Frog
41	26.4	43.7	1.5520	.3422	22.0	Frog
42	25.0	41.5	1.9924	.4173	20.9	Frog
43	23.5	24.3	2.4662	.3052	12.4	
44	24.9	29.1	3.2553	.4751	14.6	
45	23.2	27.3	2.1000	.2706	12.9	
46	19.9	31.9	2.1769	.3321	15.2	Forelegs about to emerge
47	22.3	36.7	1.3798	.2598	18.8	Second day tail resorption
48	25.5	40.7	1.8500	.3306	17.9	Tail resorption
49	24.4	36.3	1.9000	0.3378	17.8	Tail resorption

* Foot failed to develop.

TABLE IV

NET WEIGHT AND TOTAL ASH OF TADPOLES OF *Rana palustris* AT DIFFERENT BODY AND HIND-LEG LENGTHS

Prep. No. Series M	No. of Animals	Average Body Length (Millimeters)	Average Hind-Leg Length (Millimeters)	Total Net Weight (Grams)	Total Ash Weight (Grams)	Ratio Ash to Net (per Cent)
17.....	5	11.7	Bud	1.1402	0.0080	0.7
18.....	5	14.1	Bud	1.8932	.0133	0.7
29.....	2	20.1	6.6	2.2624	.0202	0.9
30.....	2	21.4	14.5	3.2841	.0331	1.0
32.....	1	24.0	20.1	2.4020	.0245	1.0
34.....	1	21.4	34.3	2.6180	.0288	1.1
41.....	1	26.4	43.7	1.5520	.0302	1.9
42.....	1	25.0	41.5	1.9924	0.0338	1.7

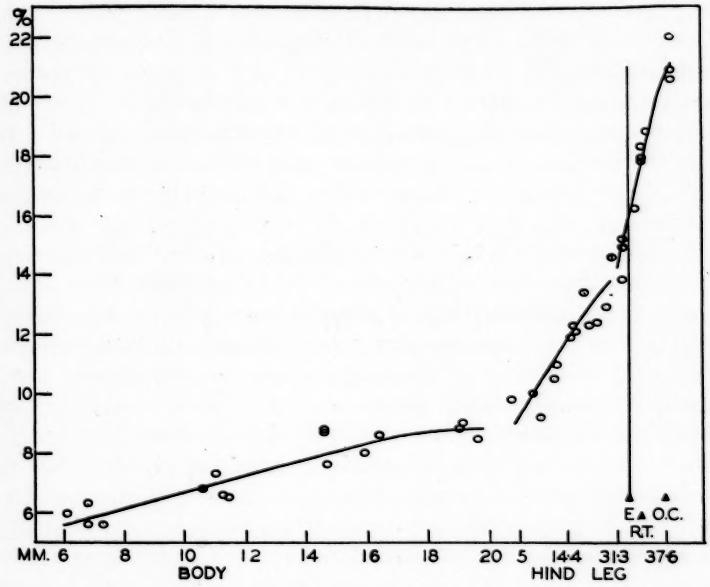

FIG. 5.—Curve showing the rate of change of the percentage ratio of dry weight to net weight during the development of tadpoles of *Rana palustris*. The abscissa is so arranged as to convert length measures into time values. Further explanation in the text. E = emergence of the forelegs; O. C. = closure of the opercular openings; R. T. = resorption of the tail.

scribed and figured in the second section of this paper. It will be noted that this rule begins with the growth of the hind legs and is, therefore, inadequate to cover all stages of the animals used for the desiccation work. If, however, it is assumed that the growth of the body was uniform during the premetamorphic period covered, an approximate abscissa can be constructed for this early period, since the necessary dates are known. The error of such a procedure would not lie in the range covered by the points nor in the general slope of the line, but only in their positions relative to each other along the abscissa. Further, the error applies only to that portion of the curve preceding metamorphosis.

An examination of such a curve, presented in Figure 5, reveals that there is a slow rise in the percentage of dry weight from the stage of 6 mm. body length to the beginning of metamorphosis. This result checks well with the work of Dempster (1930) on *Ambystoma* and of Davenport (1897) and Schaper (1902) on frogs. Of greater interest, however, is the very rapid rise in percentage of dry weight which sets in with the beginning of growth of the hind legs and continues (with perhaps a slight decline) until the time of the metamorphic climax. During the climax an even more rapid rise in dry weight occurs.

From the data it is seen that during the period of active growth of the hind legs, occupying about 18 days (See Table II and Figure 2), the proportional loss of water is much greater than during the previous developmental period, occupying about 70 days; while during the succeeding metamorphic climax, covering about 5 days, the water loss is doubled. The developmental history may then be divided into three stages as regards rate of desiccation: (1) the tadpole stage previous to metamorphosis, during which water loss is slow; (2) the prometamorphic stage, characterized by rapid growth of the hind legs with accompanying rapid decrease in water content; and (3) the metamorphic crisis, with still more rapid desiccation.

2. RANA CLAMITANS AND R. CATESBEIANA

In repeating this work on *Rana clamitans* and *R. catesbeiana*, the material was derived and treated as noted above, except that the animals were not kept under laboratory conditions for more than 3

days, and usually were treated on the day after collection. A further exception was made in keeping specimens for the completely metamorphosed stages for several weeks, since this was necessary for the determination of their metamorphic age.

At first it was thought that the limitation of material imposed by the use of animals coming from the same pond would not be serious, as the animals were larger and the greater accuracy of the technique would, therefore, make for more constant results. This was not found to be the case for the bullfrog, but on the contrary a decidedly greater variation in the results was found for this form. However, the examination of the results given in Tables V and VI leaves little doubt that the three essential phases in desiccation as noted above are operative in these two forms. In the green frog the suggestion of a sigmoid form for the curve of the rate of desiccation during prometamorphosis is even more definite than in *R. palustris*.

It is interesting to note that the percentage of dry weight in the three species at the various stages of development is not far different, but that the larger animal tends toward the lower proportion of dry weight.

D. BODY WEIGHT CHANGES

In the studies of the green frog and bullfrog, records were kept of the weight of the animals throughout the period of observation. The purpose of this study was to ascertain whether any changes in the growth rate occurred in relation to metamorphosis. The change in environment, the variation in temperature, and other factors, however, contributed to making the general results very variable for the earlier stages. It is, therefore, impossible to state whether any change in the rate of growth is correlated with the onset of prometamorphosis. Figure 3 does show a case in which there was a clear acceleration of growth prior to the beginning of prometamorphosis. However, this is decidedly the exception, and all the twenty-four other graphs show little or no effect of the advent of prometamorphosis. For the most part, the other curves are like that in Figure 4, in which the effect is probably due to the change of conditions involved in bringing the animals into the laboratory.

All the twenty-five available growth curves are consistent, however, in showing that during the major part of the prometamorphic

period growth is rapid but that a few days before the emergence of the forelegs a drop in weight begins and becomes rapid by the time the forelegs emerge.

TABLE V

NET WEIGHT AND DRY WEIGHT OF TADPOLES OF *Rana clamitans* AT DIFFERENT BODY AND HIND-LEG LENGTHS

The first group of premetamorphic animals are arranged according to body length.

The second group, consisting of animals in prometamorphosis and early metamorphic climax, are arranged according to increasing hind-leg length.

The third group of animals in the metamorphic climax is arranged according to the sequence of metamorphic events.

The abbreviations used are the same as in Figure 1. The plus sign indicates the number of days after the event.

Specimen No. Series GG	Body Length (Millimeters)	Hind-Leg Length (Millimeters)	Net Weight (Grams)	Dry Weight (Grams)	Ratio Dry to Net (per Cent)	Notes
5	15.5	1.6	0.5045	0.0485	9.6	
4	18.0	2.2	1.0221	.1103	10.8	
2	18.5	1.4	0.8365	.0857	10.2	
3	18.5	1.9	0.7862	.0670	8.5	
11	19.4	1.1	0.9288	.0934	10.1	
6	19.8	2.0	1.0917	.1253	11.5	
13	20.6	1.0	1.1605	.1315	11.3	
12	23.4	4.0	1.9325	.2095	10.8	
14	23.8	2.3	1.4977	.1787	11.9	
21	24.6	3.8	2.1248	.2398	11.3	
10	24.7	5.8	2.6608	.3538	13.3	
28	25.7	7.2	1.9840	.2463	12.4	
1	27.0	7.6	2.5172	.3033	12.0	
15	28.0	9.1	2.8493	.3585	12.6	
16	28.1	11.4	2.7807	.3466	12.5	
8	26.2	15.0	2.7666	.3851	13.9	
23	26.6	16.6	2.5170	.3412	13.6	
9	26.0	16.9	2.9180	.4158	14.2	
27	24.5	18.3	2.3904	.3210	13.4	
26	24.5	22.0	2.2972	.3192	13.9	
17	27.4	23.4	3.2331	.4446	13.8	
18	27.3	28.4	3.6293	.5213	14.4	
19	28.5	28.4	4.2291	.6062	14.3	
24	25.3	28.7	2.4341	.3354	13.8	S. W. F. L.
25	24.9	32.2	2.5715	.3453	13.4	E+1
32	24.3	33.9	2.2926	.3614	15.8	E+2
29	24.9	37.0	2.5329	.3941	15.6	T. R.
22	27.5	47.3	2.5670	.4823	18.8	T+2
30	24.6	40.4	1.3973	.2492	17.8	10 days post-met.
31	24.5	39.4	1.2715	.1667	13.1	3 weeks post-met.
33	28.2	46.4	2.1309	0.3887	18.2	4 weeks post-met.

If the desiccation curves given in the previous section are considered, it becomes apparent that this reduction in weight must be due, in part, to other factors than the loss of water, since the rate of

TABLE VI

NET WEIGHT AND DRY WEIGHT OF TADPOLES OF *Rana catesbeiana* AT DIFFERENT BODY AND HIND-LEG LENGTHS

(Arrangement as in Table V)

Specimen No. Series BG	Body Length (Millimeters)	Hind-Leg Length (Millimeters)	Net Weight (Grams)	Dry Weight (Grams)	Ratio Dry to Net (per Cent)	Notes
2.....	29.5	2.0	4.4311	0.4334	9.8	
3.....	32.3	1.9	6.6561	.6352	9.5	
5.....	32.5	1.8	6.3556	.6579	10.4	
6.....	32.6	2.1	6.7448	.6957	10.3	
7.....	35.0	2.6	8.0181	.7951	9.9	
9.....	36.1	3.0	8.4871	.8759	10.3	
8.....	37.5	4.2	9.4614	0.9524	10.1	
13.....	40.4	4.5	10.2000	1.2346	12.1	
10.....	40.5	7.4	12.6871	1.1390	9.0	
1.....	45.8	8.9	18.8175	2.0975	11.1	
4.....	41.8	11.3	13.5360	1.4050	10.4	
18.....	41.5	18.4	11.3990	1.3965	12.3	
19.....	39.3	33.7	10.3922	1.1622	11.2	
15.....	43.3	34.8	14.5850	2.1122	14.5	
11.....	43.5	44.1	16.2171	2.0300	12.5	
16.....	47.4	50.6	17.3330	2.5570	14.8	Early S. W. F. L.
20.....	43.1	54.6	15.9230	2.1112	13.3	E.—1
21.....	40.7	55.4	13.3150	1.7100	12.8	
12.....	45.2	68.5	19.4551	2.7661	14.2	T. R.—1
14.....	46.3	65.6	18.6585	2.7626	14.8	T. R.—1
17.....	35.5	64.3	8.1380	1.4396	17.7	T.—1
22.....	40.7	62.4	7.9203	1.4418	18.2	T.—1
23.....	41.5	69.1	6.4875	1.1265	17.4	About 2 weeks post-met.
24.....	41.5	.....	6.2928	1.1956	19.0	About 2 weeks post-met.
25.....	33.6	.....	3.1918	0.5643	17.7	About 2 weeks post-met.

desiccation is not greater just before the emergence of the forelegs than earlier in prometamorphosis. On the other hand, the rapid loss of weight in the later metamorphic climax is undoubtedly due to the rapid desiccation that then takes place. It seems likely that the initiation of the weight loss is due mostly to reduction in the intestinal contents.

As this paper was sent to press, the careful work of Adolph (1931)

on growth in tadpoles appeared. It is interesting to note that under well-controlled conditions this author has found that a tadpole reaches its maximum weight a week or two before the emergence of the forelegs, and thereafter the weight declines as has been described above. To judge from the figures he gives, especially Figure 1, no particular alteration in the growth rate of the body accompanies the onset of rapid hind-leg growth, i.e., prometamorphosis.

DISCUSSION

In the previous discussion, I have used the terms "prometamorphosis" and "metamorphic climax" for their descriptive convenience. A review of the data presented in this paper, however, indicates that they are really distinct phases in these species. The first, or prometamorphic, phase is characterized by rapid hind-leg growth, pigmentation change, and the second phase of desiccation as described above. If, in addition, we consider the growth of the thyroid gland as described by Allen (1919) in *Bufo* and myself (Etkin, 1930) in *Rana pipiens*, we would include the period of rapid growth and differentiation of the thyroid gland in the prometamorphic period. It may be remarked that the growth curve found by these authors for the thyroid is essentially similar to that given here for the hind legs.

The metamorphic climax beginning externally with the breaking-through of the forelegs includes that complex of resorption and developmental changes in the digestive tract, gills, and tail which are the most striking characteristics of anuran metamorphosis.

A comparison of the three species, however, shows that the resorption phenomena of the metamorphic climax cannot be said to be sharply marked off from prometamorphic phenomena. Such initial regressive changes as the resorption of the anal canal piece and of the formation of the skin window for the forelegs take place in the latter part of what has been called above the "prometamorphic period." Yet, it is apparent that, aside from this overlapping of the late prometamorphosis and the earliest climax, the two periods are fairly distinct, prometamorphosis being a period of growth activity, the metamorphic climax of destructive and remolding changes.

The primary purpose of this work is to provide the descriptive basis for an experimental analysis of the way in which the various

processes of metamorphosis are induced and correlated. That the considerations of the externally visible events discussed in this paper are only the beginnings of such an undertaking is evident. Yet from the descriptions above, a clearer idea of what changes the process of metamorphosis includes and how these changes are linked together to produce the normal sequence of events can be secured. Further, the possibility of seriating tadpoles with regard to the progress of metamorphosis and time, by means of the "abscissa rule" makes greater precision in the study of internal changes possible. By this means it is hoped to make an adequate analysis of the nature of the changes in metamorphosis and the factors that are responsible for their production.

In correlating this descriptive material with the growth and development of the thyroid as mentioned above, we find definite indications as to the *modus operandi* of the hormonal factors involved in metamorphosis. A provisional working hypothesis that is suggested for the thyroid factor may be briefly stated as follows:

1. The thyroid does not normally induce prometamorphosis; rather, its growth is to be regarded as one element of that process.

2. The growth and differentiation of the thyroid during prometamorphosis indicates that it may play a rôle as a causal factor, acting then in relatively low concentrations upon tissues of low thresholds of sensitivity to the hormone.

3. A high concentration of the thyroid secretion is the principal factor inducing the metamorphic climax. The tissues that react to it, then, have a relatively high threshold of sensitivity. It is possible that the spacing of events is determined in part by differences in threshold or latent period in different structures.

An extended review of the experimental literature from this standpoint does not seem to be warranted at the present time, but I believe enough has been said to demonstrate that a quantitative analysis of the phenomena of metamorphosis is highly suggestive and perhaps necessary for the experimental analysis of the factors regulating anuran metamorphosis.

SUMMARY

This paper describes the initial results of an attempt to furnish an exact and adequate descriptive basis for the experimental analysis

of the phenomena of metamorphosis in *Rana*. *Rana palustris*, *R. clamitans*, and *R. catesbeiana* were used.

Metamorphosis is found to be divisible into two periods which are characterized as follows:

1. *The prometamorphic period.*—Growth of the hind legs and thyroid is very rapid, the tissues undergo a distinctive phase of desiccation, and the pigment pattern changes from that of the tadpole to that of the frog.

2. *The metamorphic crisis.*—Here occurs the complex of changes involving resorption and replacement in mouth parts, gills, tail, and intestine. The desiccation of the tissues is carried through the third and most rapid stage. The thyroid and hind legs grow slowly.

Tables and charts showing the time relationships of the incidents of metamorphosis, of the rate of growth of the hind legs, and of the rate of desiccation in the three species are given.

A working hypothesis of the relation of the thyroid to the events of metamorphosis is proposed.

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THE EFFECT OF HIBERNATION AND SEASONAL VARIATION OF TEMPERATURE ON THE RESPIRATORY EXCHANGE OF FORMICA ULKEI EMERY[1]

(Four figures)

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INTRODUCTION

HIBERNATION is a phenomenon of animal life which has long been recognized to be of fundamental importance and which has consequently attracted a large number of investigators. Aristotle observed hibernation in certain animals, both vertebrates and invertebrates. Gessner (1550) and Buffon (1749) recorded more extensive observations of the hibernating behavior of various animals. During the last century a large number of investigators have studied both the natural history and the physiological aspects of hibernation. A large amount of this work has been reviewed by Krogh (1916) and Holmquist (1925), so that it is unnecessary to repeat here. However, a study of some of the more outstanding works by Spallanzani (1803), M. Hall (1832), Barkow (1846), Valentin (1857–88), Schulz (1879), Pembrey (1898), Nagai (1909), Polimanti (1912), and Rasmussen (1916) gives us a conception of the significance and effects of hibernation. These observations were made principally upon hibernating mammals such as the marmot, hedgehog, bat, or dormouse, but also to some extent upon amphibians and reptiles and more rarely upon invertebrates including mollusks, crustaceans, and insects. The background furnished by these papers serves as an introduction to the present study and may be summarized briefly as follows:

In hibernation all organisms are more or less quiescent, both physically and physiologically. Heart rate is reduced from as much as several hundred beats per minute in small mammals to 10–20 beats

[1] This investigation was undertaken at the suggestion of Professor W. C. Allee, to whom the author wishes to express his appreciation for much valuable advice and friendly interest.

per minute; rate of respiration is reduced in similar proportion. Although some hibernating animals feed at intervals, all digestive and excretory processes are markedly reduced. Growth is greatly retarded or completely interrupted during hibernation, and accompanying this there is inhibition of sexual maturity and reproduction. Nervous sensitivity is greatly reduced. The body temperature indicates a decrease in heat regulation and varies within a few degrees of the environmental temperature. Even hibernating mammals lose effective temperature regulation, and the body temperature tends to approach that of the environment. In general, hibernation involves great reduction of all metabolic processes. Evidence of this is revealed in the greatly reduced gaseous exchange and the low respiratory quotient (R. Q.) which are recorded for the various animals studied.

As mentioned above, these observations were made primarily on mammals, reptiles, and amphibians. However, recognition of the phenomenon among invertebrates, especially insects, appeared as early as 1805, and interest in insect hibernation has continued steadily to date. Recently, Parhon (1909), Bodine (1923), Fink (1925*a*), and Holmquist (1928) have presented quantitative data on the physiological effects of hibernation on bees, grasshoppers, a potato beetle, and an ant, respectively. In the work on ants Holmquist used *Formica ulkei*, so that some preliminary data on respiration in his 1928 paper serve as a point of departure for the present investigation. It has been found that the general physiological effects of hibernation on these insects are quite similar to those described for mammals and other vertebrates, i.e., a condition of greatly reduced metabolic activity occurs.

In addition to these studies of hibernation much attention has been directed during the last thirty years to the study of insect physiology in general. The effects of various environmental factors on rate of growth, rate of reproduction, and particularly on respiratory exchange, have been observed. The latter has been especially emphasized in studying the physiological condition of animals, inasmuch as the gaseous exchange and the R. Q. are interpreted as direct indicators of the intensity and nature of the metabolic processes. The large amount of literature in this field has been reviewed

by Battelli and Stern (1913) and more recently by Sayle (1928), and will be referred to in greater detail only in conjunction with the experimental results.

In these physiological studies of insects, temperature is one of the salient factors taken into account. Similarly, in any physiological study of hibernation, temperature is one of the significant factors to be considered. Especially does this appear to be true when one observes that hibernation occurs among animals in which the body temperature is entirely dependent upon the environmental temperature, i.e., among poikilothermal animals and among homoiothermal animals in which the heat-regulating mechanism is inefficient or fails to function during hibernation. Since temperature is such an important factor in the physiology of hibernation, it is the purpose of this paper to analyze the respiratory exchange of the mound-building ant, *Formica ulkei* Emery, in respect to seasonal temperature changes and its hibernating behavior. In its prosecution the work has been subdivided as follows:

1. What is the normal respiratory exchange of *Formica ulkei* during the different seasons of the year?

2. What is the effect of constant activity or constant hibernation throughout the four seasons on its respiratory behavior?

3. Are the normal seasonal changes in respiratory exchange directly correlated with temperature, or is there an inherent seasonal rhythm?

4. What are the effects of differential feeding and starvation on *Formica ulkei?*

5. What is the relation of such effects to the respiratory phenomena observed in hibernating ants?

MATERIAL AND METHODS

The experimental animals were maintained in the laboratory in large paraffin-lined galvanized iron tubs in which all natural conditions of temperature, humidity, soil moisture, food, etc., were carefully imitated. The various experimental conditions were provided in rooms of controlled temperature and humidity. Under such conditions the ants constructed typical large mounds and exhibited behavior normal for the given temperature. The ants that were kept

active at 22° C. indulged in their characteristic practice of trophallaxis, and normal reproduction took place with several queens present in each colony. Those that were kept at 4° C. remained inactive and aggregated in the galleries as in hibernation in nature. The respiratory behavior of the laboratory colonies was found to be similar to that of animals brought directly from the field nests. This imitation of natural conditions is important in all stocks kept for physiological experimentation, and should be given more consideration than it has received in many earlier investigations.

The method adopted for the measurement of the gaseous exchange of the ants is a micro-respirometer method originally described by Harrington and Crocker (1923). A modification of the animal chamber of this apparatus for use with the ants was described by Holmquist (1928). Several further modifications have been adopted which must be mentioned here. The mercury manometer was replaced by a kerosene manometer made of smaller bore tubing, about 1.5 millimeters in diameter, in order to increase the sensitivity of the apparatus. A correction was introduced into the calculation to deduct the volume of CO_2 normally present in the atmosphere (.03–.04 per cent). A molar solution of KOH was used for the absorption of carbon dioxide. The remainder of the use of this method was according to the directions given by the authors. The volumes of the gases were calculated according to the formula

$$V_1 = F \times V \times P ,$$

in which V_1 is the volume of the gas to be calculated, V is the volume of the apparatus minus the volume of experimental material, P is the barometric pressure, and F is a factor for conversion to standard conditions. The principal advantage of this type of respirometer is that it permits the simultaneous determination of both carbon dioxide and oxygen. Consequently, the significance of differences in the gaseous exchange can be indicated by the changes in the value of the respiratory quotient (R. Q. $= CO_2/O_2$). In addition, it is a convenient piece of apparatus, easy to build and repair and easy to operate.

A uniform type of experiment measuring the respiration of 25 workers for 105 minutes at a constant temperature, as indicated in the tables, was used throughout. This number of animals and length

of time was found to provide optimum normal conditions of activity and crowding of the ants.[1] Experiments were conducted at 4° C. on hibernating animals and at 22° C. on active animals. These temperatures were selected to correspond to average winter and summer temperatures for the ant mounds (Holmquist, 1928). During spring and fall, experiments were conducted at several intermediate temperatures—7°, 10.5°, 15.5°, and 18.5° C.—as shown by the tables. For the sake of compactness in the tables, the figures given do not represent single experiments but are in each case an average of from three to six individual experiments performed on and near the date given. All volumes of carbon dioxide and oxygen are given in cubic millimeters and all temperatures in degrees Centigrade.

EXPERIMENTAL RESULTS

1. SEASONAL RESPIRATORY EXCHANGE

In order to properly evaluate the effect of temperature upon the respiratory behavior of *Formica ulkei*, it was first necessary to have an accurate record of the normal seasonal gaseous exchange. Experiments measuring the gaseous exchange of ants taken from a colony maintained outdoors at the laboratory were performed at intervals of 2 to 3 weeks from February, 1930, to April, 1931. Table I summarizes these results, as described above. The accompanying graph, Figure 1, clearly shows the seasonal fluctuations which occur in gaseous exchange and the close correlation which they bear to the temperature at which the exchange was measured. In the winter during hibernation the gaseous exchange was low, 55 cu. mm. of CO_2 to 110 cu. mm. of O_2, with an R. Q. of about .500. As the temperature rose during the spring, the respiratory rate increased, with an attendant rise in R. Q. In the summer the gaseous exchange was high—for instance, 277 cu. mm. of CO_2 to 314 cu. mm. of O_2 with an R. Q. of .882. As the temperature fell in the autumn, the respiratory

[1] The ants were collected from the nests by allowing them to crawl on strips of filter paper, which were then dropped into large flasks. By cooling the flasks for 15 minutes, the ants were rendered inactive long enough to place 25 of them in one of the animal chambers. Another 15 minutes was allowed for the animal chamber and ants to return to room temperature. Such handling was necessary to prevent abnormal disturbance of the ants, which is always accompanied by the exudation of formic acid, the fumes of which are fatal to the ants in a closed space.

exchange and the R. Q. declined until hibernating conditions were restored.[1] Similar experiments were performed seasonally on ants brought directly from field nests (Table I), and the same seasonal

TABLE I

RESPIRATORY EXCHANGE—SEASONAL—1930–31

(25 Animals—105 Minutes)

Laboratory Control Ants					Field Ants				
Date	Temp. (Degrees Cent.)	Cmm. CO_2	Cmm. O_2	R. Q.	Date	Temp. (Degrees Cent.)	Cmm. CO_2	Cmm. O_2	R. Q.
2/6/30	4	55	110	0.500	2/4/30	4	56	111	0.504
2/25	4	52	105	.495					
3/3	6	54	85	.636					
4/15	10.5	101	151	.668					
5/13	15.5	128	180	.711					
5/27	18.5	176	225	.782					
6/6	22	228	260	.876	4/30	22	236	270	.874
6/27	22	254	296	.858					
7/12	22	262	306	.856					
8/12	22	277	314	.882	8/13	22	279	320	.871
8/20	22	218	243	.897					
9/25	22	265	303	.874					
10/20	18.5	165	208	.796	10/17	18.5	165	211	.782
10/27	15.5	141	201	.701					
11/9	10.5	112	174	.643					
12/1	7	69	121	.570					
1/12/31	4	55	107	.514	11/25	4	53	104	.509
1/30	4	57	119	.479					
2/2	10.5	55	110	.500*	2/6	10.5	64	117	.547*
3/10	4	55	107	.514	2/9	4	55	108	0.509
4/14	10.5	104	161	.644					
4/25	15.5	123	173	0.711					

* Compare with entry for 2/2 and 10.5° in Table VIII.

fluctuation was observed. The close correlation of the two sets of data in conjunction with the observation of normal activity and behavior indicate that this is the typical annual cycle for *Formica ulkei*.

2. RESPIRATORY EXCHANGE AT CONSTANT TEMPERATURES

Parallel series of experiments conducted with animals taken from colonies maintained at constant temperatures throughout the year

[1] The temperature at which the experiments were conducted, as indicated in the tables, are more significant than the dates, which merely indicate the seasonal sequence. In spring and fall certain temperatures between 4° and 22° C. were selected (Tables I, VII, and VIII) at which experiments were conducted to show the progress of exit from and entrance to hibernation.

showed that under such conditions the respiratory behavior remains unchanged (Table II and Fig. 2).[1] When held at a constant temperature of 22° C., the ants maintained the high rate of respiration normal for summer throughout the winter, with an R. Q. between

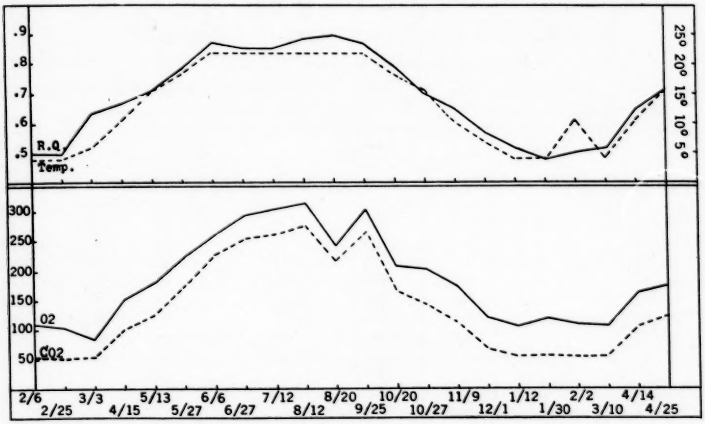

FIG. 1.—Graph for Table I showing seasonal gaseous exchange of ants. Ordinates are cubic millimeters of gas in lower half, and R. Q. on left and temperature on right in upper half. Abscissas are dates of experiments. Solid line is O_2 below, R. Q. above. Broken line is CO_2 below, temperature of the experiment above.

.800 and .900. The fact that the ants not only maintained their activity all winter but also reproduced normally in February and March is interpreted as additional evidence that their physiological condition during the winter, when subjected to the high temperature, was the same as it is normally during the summer. On the other hand, when a constantly low temperature was maintained, the ants continued to hibernate all summer, with a respiratory rate and R. Q.

[1] When these data were analyzed according to Student's Method for comparing two sets of unpaired experiments, the difference between the CO_2 production of hibernating and active ants was found to be highly significant statistically. The calculation gave a value of $t = 7.24$, which is equivalent to a probability of less than .00000001. The difference between the O_2 consumption of hibernating and active animals and the differences in gaseous exchange between normal and starved animals shown later are of similar magnitude, so that no further statistical analysis was deemed necessary.

similar to those observed normally in the winter.[1] There was no seasonal effect on either the volume or the character of the respiratory exchange when the ants were kept at either a constant high or a constant low temperature.

TABLE II

RESPIRATORY EXCHANGE—CONSTANT TEMPERATURE

(25 Animals—105 Minutes—22° and 4° C.)

CONSTANT ACTIVITY—22° C.				CONSTANT HIBERNATION—4° C.			
Date	Cmm. CO_2	Cmm. O_2	R. Q.	Date	Cmm. CO_2	Cmm. O_2	R. Q.
12/8/29	244	280	0.871	12/6/29	55	110	0.500
1/24/30	249	287	.867	1/20/30	69	136	.507
2/10	187	223	.838	1/31	41	76	.540
3/10	212	241	.879	2/23	45	89	.505
4/25	241	271	.889	3/6	48	95	.505
5/1	262	323	.842	4/3	43	89	.483
6/10	266	294	.904	4/29	57	111	.513
6/18	219	242	.904	5/18	51	97	.525
7/2	233	271	.859	6/22	53	107	.495
7/10	262	317	.826	7/7	48	91	.527
7/23	257	293	.877	7/24	47	98	.479
8/12	279	320	.871	8/14	50	99	.505
8/22	271	299	.906	8/22	56	110	.509
10/22	236	275	.858	9/20	49	105	.466
10/30	256	303	.844	10/20	47	97	.484
11/13	270	331	.815	10/31	58	119	.487
12/5	287	333	.861	11/14	56	112	.500
12/23	254	287	.885	11/25	53	104	.509
1/8/31	244	275	.887	12/23	61	119	.512
1/20	201	231	.870	1/13/31	58	112	.517
1/29	242	271	.895	1/28	47	96	.489
2/9	239	284	.841	2/9	56	112	.500
4/14	271	304	0.891	4/15	50	100	0.500
Mean.....	247	285	0.866	Mean.....	52	104	0.500

Table III shows the mean volumes of gaseous exchange and R. Q. obtained for the various experimental temperatures. It is evident that the volume of gaseous exchange and the R. Q. increase directly with the temperature. The reduction in volume of gaseous exchange observed during hibernation amounts to 79 per cent in the case of the CO_2 and 63 per cent for the O_2. Inasmuch as the CO_2 is re-

[1] Somewhat greater variation occurred in the volumes of gaseous exchange observed at 22° C. than at 4° C., owing to the fact that spontaneous activity is much more variable in the case of the active ants.

duced more than the O_2, there follows the lowering of the R. Q. from a mean of .866 at 22° C. to a mean of .500 at 4° C. The right-hand division of Table III gives the gaseous exchange in cu. mm. of gas

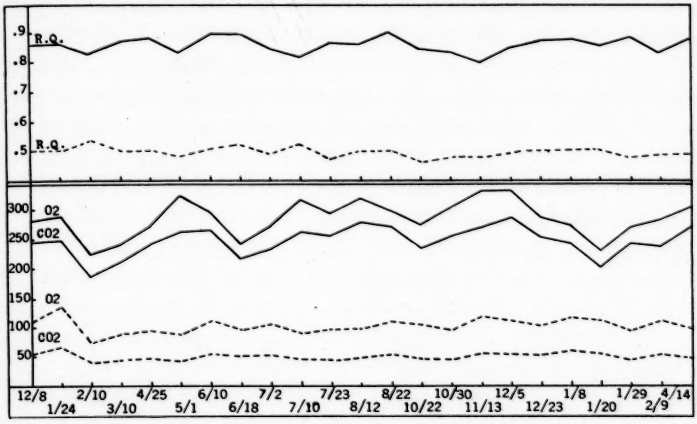

FIG. 2.—Graph for Table II showing gaseous exchange of ants at constant temperature. Ordinates are cubic millimeters of gas in lower half, and R. Q. in upper half. Abscissas are dates of experiments. Solid lines indicate experiments at 22° C. Broken lines indicate experiments at 4° C.

TABLE III

STANDARD RESPIRATORY EXCHANGE

(Cubic Millimeters per Gram per Hour)

TEMP. (DEGREES CENT.)	CMM. PER .192 GR. PER 105 MIN.			CMM. PER GR. PER HR.		
	Cmm. CO_2	Cmm. O_2	R. Q.	Cmm. CO_2	Cmm. O_2	R. Q.
4.0.....	52	104	0.500	154.75	309.50	0.500
7.0.....	62	103	.601	184.51	306.53	.601
10.5.....	104	162	.641	309.50	482.11	.641
15.5.....	135	191	.706	401.76	568.42	.706
18.5.....	171	217	.788	508.89	645.79	.788
22.0.....	247	285	0.866	735.07	848.16	0.866

per gram body weight per hour to aid in the comparison with the results of other workers which are similarly given. The experimental results presented thus far afford some interesting comparisons with those of numerous investigators working with different insects.

3. RESPIRATORY EXCHANGE OF OTHER INSECTS

In a similar seasonal study of the respiratory exchange of bees, Parhon (1909) found that a relatively constant oxygen consumption of about 20,000 cu. mm. per gram per hour took place throughout the year. Further, the greatest volume of gaseous exchange was at 10° C., so that the curve of gaseous exchange for the bees at various temperatures from 0° to 32° C. resembles that of a homoiothermal animal. Parhon attributed this phenomenon and the seasonal constancy to the social heat regulation of the bee colony by which the hive temperature is normally kept at 30°–32° C. throughout the year. Obviously, if temperature is an important factor affecting the respiratory rate, such a result is to be expected in contrast to the fluctuating seasonal exchange of the ants which are living in the mounds with the temperature at a mean of 22° C. in the summer and 4° C. in the winter (Holmquist, 1928). In contrast to the constant exchange of the bees, Parhon found that the O_2 consumption of flies increased with the temperature from 0 cu. mm. per gram per hour at 10° C. to 14,000 cu. mm. at 37° C. In all experiments on both bees and flies, the CO_2 production was very nearly equal to the O_2 consumption, so that the R. Q. fluctuated around 1.0.

Vansell (1929) observed a relatively constant CO_2 production of 13,000–15,000 cu. mm. per gram per hour for bees in summer and winter. Battelli and Stern (1913) reported that flies at 20° C. produced 2,300 cu. mm. per gram per hour of CO_2 and consumed 3,100 cu. mm. of O_2. It is to be noted that these are very high rates of respiratory exchange. Parhon, in a comparative table, pointed out that the rate for the bees greatly exceeds that of birds, various mammals, reptiles, and other animals. In general it is assumed that the respiratory exchange of insects is relatively greater than that of other animals. However, the volumes of gaseous exchange reported here for *Formica ulkei* and the results for other insects summarized below are quite comparable to the volumes given for some of the small mammals and birds, i.e., 700–1,000 cu. mm. per gram per hour. The volumes observed for several different nymphs and pupae are even lower, ranging from 350 to 500 cu. mm. per gram per hour.

Other investigators, such as Rogers (1929) and Bodine (1923), working with grasshopper nymphs; Sayle (1928), with dragon-fly

nymphs; Battelli and Stern (1913), with May beetles, silkworm moths, and flies; and Slowtzoff (1909), with manure beetles and ants, found that the volume of gaseous exchange and the R. Q. increased with the temperature and that the volumes between 0° and 5° C. were 75–95 per cent less than those measured at 20–25° C. Thus, some insects show an even greater reduction at low temperatures than that reported for *Formica ulkei*, although none of these insects show as great a reduction as that observed in the case of some hibernating mammals. Regnault and Reiset have reported that the respiration of the hibernating marmot is reduced thirty times.

Tauchert (1929) reported that mealworms, May beetles, and *Vanessa* normally consume as much as 800 cu. mm. per gram per hour of O_2. Fink (1925*a*) found that the potato beetle, when active, produces 714 cu. mm. per gram per hour of CO_2. This volume was reduced 70–80 per cent during hibernation. The R. Q. of the potato beetle also was reduced from .76 to .60 during the early stages of hibernation, although it rose again during the later stages. Frew (1929) mentioned unpublished data which indicate that the R. Q. of insects was reduced from .72–.80 during activity to .41–.71 during hibernation. Bodine (1923), studying hibernation of grasshopper nymphs, reported a CO_2 production of 398 cu. mm. per gram per hour at 18°–20° C. This was reduced to $\frac{1}{8}$–$\frac{1}{10}$ of that amount between 0° and 8° C. Regen (1906) obtained a similar reduction in the CO_2 production of a cricket. In another case, Bodine (1921*a*) found that the CO_2 output of grasshopper nymphs was 637 cu. mm. per gram per hour at 20° C. and 255 cu. mm. at 5° C., a decrease of 60 per cent. Battelli and Stern (1913) observed that silkworm moths produced 900 cu. mm. per gram per hour of CO_2 at 20° C. and that these volumes increased at 30° and 40° C. The R. Q. also increased from .64 to .69. Similar results were obtained with May beetles, which produced 610 cu. mm. per gram per hour of CO_2 at 20° C. with an R. Q. of .65. The R. Q. for the flies mentioned above was .74 at 20° C.

Slowtzoff (1909) found that the CO_2 production of manure beetles and ants increased with temperature amounting to about 400 cu. mm. per gram per hour of CO_2 at 20° C. Such a volume is somewhat lower than that reported for *Formica ulkei* and for other beetles, and

may be associated with a variation in the response of the respiratory exchange to temperature which was pointed out by Slowtzoff. He observed a temperature interval of about 12° beginning at different levels (i.e., 15°–25° C.) in different insects within which no increase in respiratory rate occurred. Similar observations were made by Vernon (1897), Schulz (1879), and Bütschli (1874). No such static period was observed in the respiratory behavior of *Formica ulkei* or in the observations on other insects described above, so that considerably higher rates of gaseous exchange were recorded for experiments at 20°–25° C., when the increase in rate extended over the whole temperature range. Slowtzoff reported an R. Q. of .8 for the beetles, .9 for the ants, and 1.0 for the flies. Regnault and Reiset, as early as 1849, found that the cockroach consumed 727 cu. mm. of O_2 per gram per hour and that the May beetle used 716 cu. mm. of O_2 with an R. Q. of .8. In general the results of these different investigators, who used a variety of insects, exhibit considerable uniformity to which the present data ascribe. With such a basis for comparison, further experimental results can now be described.

4. EFFECT OF TEMPERATURE REVERSAL ON RESPIRATORY EXCHANGE

In order to further test the effect of temperature independently of the seasons, ants were taken from the constant 22° colonies and subjected to a temperature of 4° C. for 1–2 hours. When the respiration was then measured at 4° C., the volume of gaseous exchange was reduced as it is normally during hibernation. Similarly, the R. Q. was reduced to about .500. Subsequently, when the same ants were restored to a temperature of 22° C. for 1–2 hours, they regained their normal activity, and experiments conducted at 22° C. showed a recovery of the normal rate and R. Q. for that temperature (Table IV). Conversely, when ants from the colonies subjected to constant hibernation were warmed to 22° C. and the respiration was measured, the same type of reversal occurred. In this case the volume of gaseous exchange increased, and the R. Q. was raised to .800 to .900. Evidently the respiratory rate and the R. Q. are functions of the experimental or environmental temperature. Kayser and Gingliger (1927), experimenting on non-hibernating animals such as the tortoise, mouse, rabbit, pigeon, and guinea pig, have expressed this

same opinion. They observed an increase in R. Q. with increase in experimental temperature and interpreted the R. Q. as a function of temperature, considering it as a factor independent of muscular activity or digestive processes. The R. Q. obtained represents the algebraic sum of the R. Q. of each of the several oxidative processes going on at any one time. The equilibrium of these oxidative processes varies with temperature just as it does with change in concentration of the cellular elements. Consequently, the R. Q. varies with the oxidative processes according to temperature. Such facts seem quite in accord with the present data and thoroughly acceptable in view of the current physiological interpretations of the R. Q. as an indicator of the character of metabolic processes.

TABLE IV

RESPIRATORY EXCHANGE—TEMPERATURE REVERSAL

(25 Animals—105 Minutes)

NORMAL ANTS				HIBERNATING ANTS			
	Cmm. CO_2	Cmm. O_2	R. Q.		Cmm. CO_2	Cmm. O_2	R. Q.
Exp. at 22° C.	244	275	0.887	Exp. at 4° C.	50	98	0.510
Exps. at 4° C.	42	75	0.560	Exps. at 22° C.	195	203	0.960
	57	105	.542		280	317	0.883
	37	66	.616		196	194	1.01
	19	37	.513		203	260	0.780
	37	54	.685		256	318	.805
	32	56	.571		225	274	.821
	46	107	.430		201	215	.934
	59	147	.401		206	221	.932
	35	80	.430		166	182	.912
	58	96	.604		213	248	.858
	51	126	0.404		193	201	0.960
Mean.....	43	86	0.498	Mean.....	212	239	0.886
Same at 22° C.	226	258	0.876	Same at 4° C.	54	103	0.524

5. EFFECT OF DIFFERENTIAL FEEDING ON RESPIRATORY EXCHANGE

A large number of investigators have shown that the R. Q. of mammals varies with the diet in such a manner that the R. Q. for a carbohydrate diet is approximately 1.0; for protein, .80; for fat, .70 (Krogh, 1916). When three like colonies of *Formica ulkei* kept at

22° C. were subjected to such differential feeding, the R. Q. was similarly observed to be an indicator of the character of the metabolism (Table V). The experiments on the carbohydrate-fed ants showed an R. Q. fluctuating around 1.0 with a mean of .984. Parhon (1909) found that bees (which were living on a carbohydrate diet) also exhibited an R. Q. of about 1.0. In the case of the ant colonies fed on fat and on protein, the R. Q. in the beginning, .63 and .71, respectively, was indicative of the diet; but subsequently the effect

TABLE V

RESPIRATORY EXCHANGE—DIFFERENTIAL FEEDING*

(25 Animals—105 Minutes—22° C.)

DATE	CARBOHYDRATE†			FAT			PROTEIN		
	Cmm. CO_2	Cmm. O_2	R. Q.	Cmm. CO_2	Cmm. O_2	R. Q.	Cmm. CO_2	Cmm. O_2	R. Q.
7/24/30	290	314	0.923	175	276	0.634	138	194	0.711
8/1	243	231	1.05	133	223	.596	80	155	.516
8/6	260	278	0.935	94	191	.492	106	157	.675
8/19	252	235	1.07	91	152	.598	46	95	.484
8/26	243	250	0.972	55	106	0.518	42	85	0.494
Mean..	258	262	0.984	110	190	0.579	82	137	0.600

* These colonies were starved 2 weeks and then fed the indicated diet for 1 week before these experiments were begun.

† The R. Q. expected from a carbohydrate diet is 1.0; from fat, .70; from protein, .80.

of starvation was superimposed on that of the limited diet and both the volume of the gaseous exchange and the R. Q. were further reduced.[1] As in the case of the effect of temperature, the respiratory rate and the R. Q. are indicators of the intensity and character of the metabolic activity of the ants. Under these conditions they are evidently functions of the food supply. Other investigators, namely, Slowtzoff (1909) and Gourévitch (1928), have come to this same conclusion. Slowtzoff found that the R. Q. for flies fed on sugar was 1.0. When they were fed on meat for several days, the quotient fell to .8. The latter author found that the R. Q. of the cockroach was .65–.84 after several days of starvation; .78–.83 when fed on fresh cream; and 1.01–1.07 when fed on starch.

[1] See section 6, on the effects of starvation on the volume of gaseous exchange and the R. Q.

6. EFFECT OF STARVATION ON RESPIRATORY EXCHANGE

Along with the three colonies fed on the restricted diets, a fourth colony, also kept at 22° C., was subjected to starvation. In Table VI and Figure 3, the data obtained from experiments at 22° C. on normal and starving ants are compared. It is evident that starvation causes a progressive reduction in the volume of gaseous exchange accompanied by a reduction of the R. Q. from the normal level, .800–.900, to .450, indicating a reduction in metabolic activity and an alteration in its character comparable to that observed generally in mammals, including man. After 5 weeks of starvation, the CO_2 pro-

TABLE VI

RESPIRATORY EXCHANGE—STARVED ANTS

(25 Animals—105 Minutes—22° C.)

DATE	NORMAL ANTS			STARVED ANTS		
	Cmm. CO_2	Cmm. O_2	R. Q.	Cmm. CO_2	Cmm. O_2	R. Q.
7/2/30	233	271	0.859	213	255	0.835
7/10	265	319	.830	190	267	.711
7/16	259	295	.877	156	246	.634
7/24	268	306	.875	93	157	.592
7/31	243	275	.883	75	131	.572
8/6	280	313	.894	60	112	.535
8/19	286	317	.902	56	123	.455
8/26	256	280	0.914	18	40	0.450

duction was reduced 72 per cent, while after 8 weeks it was reduced 92 per cent. At the same intervals the O_2 consumption was reduced 66 per cent and 85 per cent, respectively. Thus the reduction in metabolic activity produced by starvation is considerably greater than that produced by low temperature during hibernation.

In this connection Tauchert (1929) observed that the O_2 consumption of meal worms, May beetles, and *Vanessa*, when fasting, was lowered 50–75 per cent. Sayle (1928) reported that the CO_2 production of dragon-fly nymphs was decreased 50 per cent during 4 weeks of starvation. Davis and Slater (1926) believe that starvation depresses the R. Q., for they found that cockroaches have an R. Q. between .72 and 1.0 when fed but that the R. Q. was between .75 and .85 when the animals were starved. Fink (1925*a*) found that a month

of starvation reduced the CO_2 production of the potato beetle from 714 to 153 cu. mm. per gram per hour, about 79 per cent. Bodine (1921*a*) reported that 120 hours of starvation caused a decrease in the CO_2 production of certain adult grasshoppers from 497 to 363 cu.

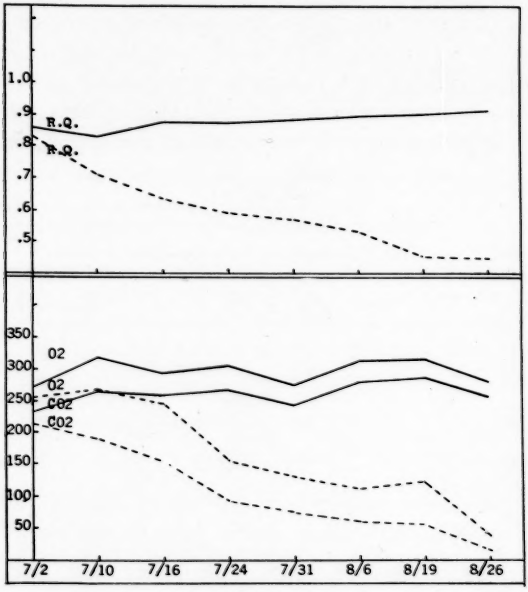

FIG. 3.—Graph for Table VI showing gaseous exchange of ants during 8 weeks of starvation at 22° C. Ordinates are cubic millimeters of gas below, R. Q. above. Abscissas are dates of experiments. Solid lines indicate experiments on normal ants. Broken lines indicate experiments on starving ants.

mm. per gram per hour, a decrease of about 27 per cent. Other experiments showed a reduction up to as much as 54 per cent. Thus the effect of starvation on the gaseous exchange of *Formica ulkei* is similar to its effect upon the metabolism of numerous other insects.

7. EFFECT OF STARVATION UPON RESPIRATORY EXCHANGE DURING HIBERNATION

During the course of the experiments certain relations between the effects of starvation and hibernation were observed. In the left-

hand division of Table VII and in Figure 4 are recounted the results of experiments on the respiration of ants during the normal exit from hibernation in the spring. Under these conditions the rate of gaseous exchange and the R. Q. increased with the rising temperature until they reached the level shown to be typical of the active ants in summer at a constant temperature of 22° C. At 10.5° C. some of the ants were segregated so as to be unable to begin feeding, which they do normally as activity increases above that temperature. Although

TABLE VII

RESPIRATORY EXCHANGE—EFFECT OF STARVATION UPON EXIT FROM HIBERNATION

(25 Animals—105 Minutes)

CONTROL-NORMAL EXIT					UNFED-ABNORMAL EXIT				
Date	Temp. (Degrees Cent.)	Cmm. CO_2	Cmm. O_2	R. Q.	Date	Temp. (Degrees Cent.)	Cmm. CO_2	Cmm. O_2	R. Q.
2/25/30	4	52	105	0.495	2/25/30	4	52	105	0.495
3/3	6	54	85	.636	3/3	6	54	85	.636
4/15	10.5	101	151	.668	4/15	10.5	101	151	.668
5/13	15.5	128	180	.711	5/8	15.5	92	161	.571
5/27	18.5	176	225	.782	5/17	18.5	94	150	.626
6/6	22	228	260	.876	5/27	22	111	185	.600
6/27	22	254	296	.858	6/8	22	221	254	.870*
7/12	22	262	306	0.856	6/17	22	157	243	0.646†

* Same ants as 5/27 after feeding 10 days.

† Same ants as 6/7 after starving again for 10 days.

the rising temperature stimulated activity, the volume of gaseous exchange and the R. Q. were reduced by the attendant starvation and remained far below the normal for the temperatures at which the experiments were conducted (right-hand division of Table VII). Later, when these ants, now at 22° C., were supplied with food, they recovered the normal respiratory behavior (entry for June 8, Table VII). Finally, subsequent starvation incited a secondary decline, as shown by the entry for June 17.

The same sort of effect of starvation was observed during the winter of 1931, when unusually warm weather raised the temperature of the mounds, both outdoors at the laboratory and in nature, to 10°–12° C. This higher mound temperature was accompanied by

an increase in the activity, and consequently in the metabolic rate, of the ants in hibernation. However, no feeding took place, and experiments performed at 10.5° C. (Table VIII) did not show the expected increase in the respiratory activity or R. Q. The latter re-

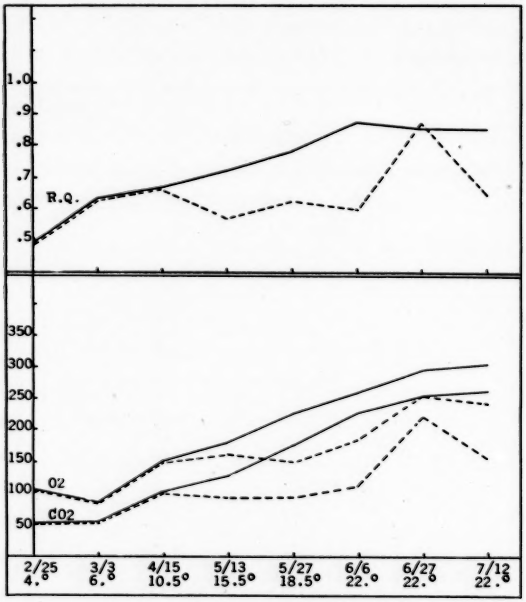

FIG. 4.—Graph for Table VII showing gaseous exchange of ants during exit from hibernation. Ordinates are cubic millimeters of gas below, R. Q. above. Abscissas are dates and temperatures of experiments. Solid lines indicate experiments during normal exit. Broken lines indicate experiments during abnormal, starved exit.

mained around .500, as in the case of ants normally hibernating at 4° C. Further, when these ants were warmed to 22° C., the gaseous exchange and the R. Q. were still below normal, as in the case of the ants starved during the exit from hibernation in the spring (Table VII). Experiments performed on ants brought directly from the field yielded analogous results (right-hand division of Table VIII). However, when these ants were fed and subsequently tested, the results showed a recovery of the respiratory behavior shown to be typical

of 10.5° C. (entry for February 24, Table VIII). Later in the winter when cold weather had restored the mound temperature to the normal level, experiments, conducted as usually at 4° C., revealed the characteristic low rate of respiration with an R. Q. about .500, which was shown to be typical of hibernation. Finally, some experiments performed at 10.5° C. on ants taken at this time from the colony subjected to constant hibernating conditions showed a normal increase in respiration in conjunction with the increase in temperature. Ap-

TABLE VIII

RESPIRATORY EXCHANGE—EFFECT OF STARVATION DURING HIBERNATION

(25 Animals—105 Minutes)

LABORATORY CONTROL ANTS					FIELD ANTS				
Date	Temp. (Degrees Cent.)	Cmm. CO_2	Cmm. O_2	R. Q.	Date	Temp. (Degrees Cent.)	Cmm. CO_2	Cmm. O_2	R. Q.
1/12/31	4	55	107	0.514	2/9/31	4	55	108	0.509
1/30	4	57	119	.479	2/6	10.5	64	117	.547
2/2	10.5	55	110	.500	2/23	22	124	189	0.656
2/3	22	152	236	.644					
2/24	10.5	93	153	.607*					
3/10	4	55	107	0.514					
Normal means for each temperature	4	51	100	0.505					
	10.5	98	160	.612					
	22	239	273	0.875					

* Same ants as 2/2 after feeding 3 weeks.

parently the increased rate of metabolism during the warm weather caused the ants to undergo some starvation in the absence of food. Normally the greatly reduced metabolism accompanying hibernation protects them from such starvation. Marked variations occur in this respect in different animals. For instance, Bodine (1923) pointed out that hibernating grasshopper nymphs become active at intervals and feed and that starved individuals do not survive the period of hibernation. The torpidity of such insects in hibernation is not so great as in the case of the ants, so that their need for food is due in part to the fact that metabolic activity is not reduced to a minimum, as is that of these ants. In this connection the relative amount of food stored, in the tissues of the fat body for instance, per

individual body weight of the different insects has not been investigated, although it must be mentioned as a contributing factor.

This analysis shows that in *Formica ulkei* reduced metabolic activity occurs in both hibernation and starvation. In the former case, however, activity and metabolism are uniformly lowered from the beginning, whereas in starvation the animal remains active, depleting its reserves, and the metabolic rate progressively declines as this occurs. The ultimate result is a greater depletion than occurs in hibernation, and finally death. Fink (1925*a*) expressed the same opinion in respect to his data on the potato beetle undergoing starvation.

DISCUSSION

In the introduction, reference was made to preliminary studies by Holmquist (1928) on the respiration of *Formica ulkei* which was taken as a starting-point for the present work. Certain differences have appeared which should be mentioned. The volume of gaseous exchange reported by Holmquist is extremely low, about 138 cu. mm. of CO_2 per gram per hour at 22° C. Reference to Table III and the work of other investigators mentioned shows that such a volume is too low to be comparable to that obtained on any of the different insects studied. Two factors may be involved here. In the first place, the mercury manometer used by Holmquist is a much less sensitive instrument than the respirometer employed in these experiments. Secondly, he used a large number (100) of ants in an experiment which extended over about 24 hours. It has been pointed out above that the conditions of these experiments, 25 animals and 105 minutes, were found to be optimum for the ants. It is highly probable, then, that overcrowding of the ants may be the explanation for the very low volume of gaseous exchange recorded by Holmquist. The effect of crowding and of isolation upon insect respiration deserves more attention than it has received to date.

The R. Q. for the ants at low temperatures (3°–6° C.) was reported by Holmquist to be higher, slightly above 1.0, than that of the active ants for which he found a mean R. Q. of .888. Such an R. Q. has not been observed for any of the insects on which respiratory investigations have been reported, nor is such an R. Q. expected to result from the alterations in metabolic activity produced by low tempera-

ture. Athanasiu (1909) reported that during hibernation, the R. Q. of frogs is raised from the summer level of .77 to .97, or even to above 1.0. To explain this high quotient, he postulated that a storage of oxygen occurred in the tissues in preparation for hibernation. The use of this stored oxygen during hibernation then accounted for the small consumption observed. Iizuka (1926) did not confirm this observation for frogs during hibernation, but he reported an increase in the R. Q. up to 1.0 or even above when frogs were desiccated for 24 hours. Neither of these interpretations of an increase in R. Q. seems applicable to the present situation, as will be pointed out below. However, the high R. Q. may result from an error introduced into the calculations if formic acid fumes from the ants are absorbed by the KOH along with the CO_2. Furthermore, asphyxiation of the ants results from exposure to confined formic acid fumes. Vernon (1896) observed that asphyxia of a number of marine invertebrates is associated with an increase in the R. Q. to well above 1.0, that is, they continue to produce CO_2 when little or no O_2 is being consumed. Either or both of these conditions may be a factor in the high R. Q. which Holmquist observed for the ants in hibernation, inasmuch as the large number he used would be difficult to handle without considerable disturbance, which always produces an exudation of formic acid from the ants.

It has been pointed out that a decrease from a high rate of respiration, 735 cu. mm. of CO_2 per gram per hour, with a mean R. Q. of .866, to a low rate, 155 cu. mm. of CO_2 per gram per hour, with a mean R. Q. of .500 during hibernation, is characteristic of *Formica ulkei*. Such reactions are likewise typical of numerous other insects for which hibernation has been investigated, and similar to the effect of hibernation on mammals and numerous other animals. The small volume of gaseous exchange is direct evidence of the low rate of metabolism of the hibernating animals. But what factors are involved in this reduced rate of metabolism to produce the low R. Q. which accompanies it?

Many theories, reviewed by Rasmussen (1916), have been advanced to explain the phenomenon of hibernation; but only a few offer any real physiological explanation for the effects that are so uniformly observed, particularly the low R. Q. Two of the hypothe-

ses advanced in this connection postulate a storage of gases in the blood. Regnault and Reiset (1849) were of the opinion that O_2 is stored in the tissues during hibernation, so that the amount of oxygen intake becomes relatively greater than that actually used, and hence considerably greater than the CO_2 produced. On the other hand, Du Bois (1896) presented the theory of carbon dioxide narcosis as a cause for hibernation. This implies an accumulation of CO_2 in the tissues, so that the amount measured is less than that actually produced in the oxidative processes. In view of the volumes of gases that would necessarily be stored in order to account for the low R. Q. during hibernation, neither of these theories seems very plausible; and other inconsistencies in them have been pointed out by many investigators (Krogh, 1916; and Nagai, 1909).

Another cause of the low R. Q. described by Du Bois (1896), Weinland and Riehl (1907), and Frew (1929) is the conversion of fat into glycogen during hibernation, which produces an R. Q. of .700 or less, with the subsequent consumption of the glycogen to account for the R. Q. of about 1.0 which they observed upon the exit from hibernation. This theory is generally criticized on the ground that the amount of glycogen that would be stored is far in excess of that found in the animal body. It would seem to apply to insects only in cases where hibernation occurs during the larval period, for it is then that glycogen is found to increase in the insect body to be used subsequently during pupation (Uvarov, 1929). Only traces of glycogen are found in the adult insect, so that the theory does not seem tenable for insects such as the ants that hibernate as adults. Parhon (1909) found only .14–.21 per cent by weight of glycogen in bees. Further, Timon-David (1928), pointed out that in the hibernation of a butterfly, in which there is an extreme reduction of metabolism, the enormous fat reserve remains constant. It is used up in the transformation into the adult but not as reserve food material during hibernation.

Fink (1925*a*) found that reduced respiratory metabolism is associated with reduced catalase activity and catalase content of the hibernating potato beetle. Bodine (1921*b*) also found that a decrease in CO_2 output normally accompanied decrease in catalase in the grasshopper, firefly, and potato beetle, but that during hibernation

the marked decrease in CO_2 production was not accompanied by the reduction of catalase content. No catalase determinations have been made for the ants.

One of the most commonly observed physiologic changes attendant upon hibernation is the reduction in body water content. Numerous investigators, including Tower (1917), Bodine (1923), Caldwell (1925), and Townsend (1926), have reported a decrease in water content of various hibernating insects of from 15 to 55 per cent of the body weight. It was pointed out by Caldwell that such reduction of water content may affect the metabolic processes through accumulation of waste products, through inadequate distribution of oxygen or food materials, or by direct action in slowing down the oxidative processes. Thus it may well be a causal factor for the low R. Q. observed in hibernation accompanied by a decrease in body water content.

However, the reduction in body water content occurring in *Formica ulkei* during hibernation is considerably less than that of other insects. Holmquist (1928) reported a decrease in hibernating workers of about 5–15 per cent, while a variation of about 5 per cent was observed in the determinations of the water content of the active workers. Evidently, the reduction of water content is not so significant a factor in this case as it may be in some of the other insects. Furthermore, this dehydration appears to be associated with cold hardiness. Bodine (1923), working with two species of grasshoppers, found that *Chortaphaga australior*, which does not normally hibernate, will hibernate like *C. viridifasciata* when exposed to the same conditions, and that the physiological reactions of *C. australior* are then the same, including reduction in water content, as those of *C. viridifasciata* during hibernation. Tower (1917) observed that potato beetles, when raised in a warm desert region, do not undergo the reduction of water content during hibernation which accompanies their hibernation under conditions of low temperature. Payne (1926*a* and 1928) showed that dehydration is associated with cold hardiness of the Japanese beetle larvae and several oak borers to both the quantity and intensity factor of low temperature. In addition it is known (Babcock, 1912; and F. G. Hall, 1922) that water produced in metabolic processes, i.e., metabolic water, is an important

source of the body water content of many insects. Since the rate of metabolism is greatly reduced at low temperature, it is obvious that a concomitant reduction in metabolic water and hence in body water content would occur. Evidently, if reduction of water content is a factor in the alteration of metabolic processes and the lowering of the R. Q. during hibernation, it is intimately associated with another factor, namely, temperature.

The direct correlation of the volume of gaseous exchange and the magnitude of R. Q. to temperature which has been shown for *Formica ulkei* indicates that it is an important factor affecting the metabolic processes of the insect. In fact, the respiratory exchange and the R. Q. observed appear to be direct indicators of the environmental temperature through its effect on metabolism. Reference has already been made to a similar opinion expressed by Kayser and Ginglinger (1927) for non-hibernating mammals. They believe that the observed R. Q. represents the algebraic sum of the R. Q. of each of several oxidative processes going on at any one time, the equilibrium of which varies with temperature.

Nagai (1909), studying the metabolism of several hibernating mammals, expressed a similar opinion in a slightly different manner. He believes that oxidation intensity during hibernation varies with the body temperature and that the lowering of the R. Q. is due to imperfect and incomplete oxidative reactions caused by the lowered body temperature, and not to conversion of fat into sugar or to gas storage in the tissues. Such abnormal metabolism he found to be further substantiated by the great displacement of nitrogen distribution and the presence of lactic acid in the urine. Such a concept seems applicable to the data at hand not only in respect to the effect of temperature but also in respect to that of starvation. It has been shown that the rate of respiration and the R. Q. of *Formica ulkei* vary in respect to diet in the manner expected from the known effects of diet upon metabolic processes and that a great reduction in gaseous exchange and R. Q. is associated with starvation. However, starvation cannot be interpreted as a causal factor for the low R. Q. observed in hibernation, since it has also been shown that the inactivity and altered metabolism associated with low temperature protect these ants from starvation during hibernation. From this anal-

ysis of the physiological effects of hibernation upon *F. ulkei*, it appears that temperature is the major factor concerned in the metabolic changes which occur.

In conjunction with this discussion of the physiological effects of temperature upon these ants, it is interesting to point out how their behavior is modified in nature in response to temperature as an environmental factor. Baumberger (1914) observed that, while various factors are involved in insect hibernation, the dominant factor in any one case is the one to which the insect is most sensitive or which is nearest its vital limits. Now, it is generally admitted that not all insects hibernate in response to low temperature, as Baumberger (1917) has shown specifically for the codling moth. Some beetles begin to hibernate before low temperature sets in (Kirby and Spence, 1817–63). Others hibernate periodically as a necessary part of the life-cycle. The rest period or exposure to cold of hibernation is often essential to emergence from pupation or to further reproduction. However, many insects, such as *Drosophila*, the house fly, and the ants, hibernate in response to temperature changes. Roubaud (1925) calls such insects, which exhibit no obligatory hibernation but which show a direct correlation in activity to temperature, "homodynamic insects."

Some authors imply that the hibernation of such homodynamic forms is merely cold torpor and not true hibernation. However, if metabolic changes are a criterion of true hibernation (and it is generally believed that they are), then *Formica ulkei* is a homodynamic insect which undergoes a true hibernation and not merely cold torpor in response to low temperature. According to Baumberger, this is the factor to which the ants are most sensitive. Such an idea is compatible with ant behavior in general, as the following instances will illustrate.

The eggs, larvae, and pupae of ants are constantly moved around in the nest by the workers in response to optimum conditions of moisture and temperature. Cobelli (1903) observed that several species of ants enter upon and exit from hibernation in direct correlation to their sensitivity to cold. Miller and Gans (1925) experimentally caused ants to change their path, first positively and then negatively, in response to radiant heat of varying intensity. Steiner (1924) and

Andrews (1927) have shown that regulation of temperature occurs in ant mounds. According to Steiner, this temperature control is due to respiratory heat produced by the ants and is associated with the social life, as in the case of over-wintering bees. He terms it "social heat regulation" and indicates that it implies not only a well-developed individual temperature sense but also a social heat instinct.

With such evidence of the relation of ants to temperature in mind along with the physiological data showing the metabolic reactions to temperature, it is easy to accept the temperature mechanism which Holmquist (1928) postulated for the entrance to and the emergence from hibernation of *Formica ulkei*. The description of this process, confirmed by present observations, is as follows: As temperature declines in the fall, the lowering of the air temperature causes a similar change in the surface soil, but the deeper parts of the nest are only slightly affected. The result is a reversal of the temperature conditions in the mound, so that the lowest temperatures occur at the surface and progressively higher temperatures are found toward the soil-water level. In order to escape the low temperatures in the upper parts of the nest, the ants retire as far as possible from the colder surface soil to the warmer levels below. The arrival of ants from above and the upward migration of those from below to escape the rising water level results in the crowding of the ants into the densely packed masses typically found in the lower galleries during hibernation. Emergence in the spring is likewise a temperature phenomenon. This is shown by the fact that surface activity begins as soon as frost is out of the ground and gradually increases in intensity until all ants have left their hibernation quarters and become active at the surface. A few scattered ants that overwinter in the frozen soil of the mound are roused to activity first when the surface soil becomes warmed in the spring. In some nests, at least, the hibernating masses of ants are forced upward at about the same time by the encroaching soil water. This not only brings them nearer the warmer surface but places them within reach of the already active surface ants. It is possible that the latter may drag individuals from the hibernating masses toward the warmer surface, thus aiding and hastening the exit from hibernation.

It is evident that these references to the bionomics of ants support the experimental findings, so that we now have a functional picture of the seasonal reactions of these ants to temperature based upon the observed metabolic changes and their natural responsiveness to temperature as an environmental factor. Let us summarize the argument in a few concluding statements: First, the data show that the intensity and character of the respiratory exchange of *Formica ulkei* are fundamentally modified by changes in temperature. Second, reduction in body water content is not as significant a change accompanying the hibernation of *F. ulkei* as it is in the case of many other insects; under any circumstances, dehydration seems to be associated with cold hardiness, and consequently its physiological effects are closely tied up with those of temperature. Third, although starvation is a factor capable of producing such abnormal metabolic processes as are indicated by a low R. Q., it has been shown that hibernation of *F. ulkei* normally protects the species from starvation. Fourth, other factors which, under certain circumstances, may be associated with reduced respiratory exchange or low R. Q. are not applicable to these data. Fifth, temperature is a factor to which these ants are very responsive in nature and which appears to be sufficient as a mechanism to account for their normal behavior. Consequently, temperature must be recognized as the primary causal factor involved in the hibernation of *F. ulkei*.

CONCLUSIONS

1. The normal respiratory exchange of *Formica ulkei* is determined and is found to exhibit a seasonal rhythm.

2. Ants living under conditions of constant temperature maintain a constant rate of respiration and a uniform R. Q. throughout the four seasons.

3. The rate of respiration and the R. Q. vary directly with the experimental or environmental temperature.

4. There is a reduction of 79 per cent in CO_2 production and 63 per cent in O_2 consumption between the active ants at 22° C. and the hibernating ants at 4° C.

5. The mean R. Q. for active and hibernating ants is .866 and .500, respectively.

6. The R. Q. varies according to the limitation of the diet. It is .984 for carbohydrate, .711 for protein, and .634 for fat.

7. Starvation produces a marked reduction in the volume of respiratory exchange and in the R. Q. It is more pronounced than that produced by hibernation, i.e., 92 per cent for CO_2 and 85 per cent for O_2.

8. Normally, hibernation protects the ants from starvation during their period of inactivity at low temperatures.

9. The analogous effects of low temperature and starvation on the rate of respiratory exchange and the R. Q. of *Formica ulkei* and the inadequacy of other physiological explanations for such changes in the R. Q. indicate that abnormal and incomplete oxidations due to low temperature are the cause for the reduced metabolism occurring during hibernation.

10. Temperature is described as the primary factor involved in the hibernation of *Formica ulkei* which is shown to be a type of social homodynamic insect.

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